Bacterial cells with improved tolerance to polyamines

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(54) Title: BACTERIAL CELLS WITH IMPROVED TOLERANCE TO POLYAMINES

(57) Abstract: Provided are bacterial cells genetically modified to improve their tolerance to certain commodity chemicals, such as polyamines, and methods of preparing and using such bacterial cells for production of polyamines and other compounds.

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BACTERIAL CELLS WITH IMPROVED TOLERANCE TO POLYAMINES

FIELD OF THE INVENTION

The present invention relates to bacterial cells genetically modified to improve their tolerance to certain commodity chemicals, such as polyamines, and to methods of preparing and using such bacterial cells for production of polyamines and other compounds.

BACKGROUND OF THE INVENTION

Polyamines (NH2-R-NH2, where R is an alkyl chain) are most commonly used as precursors for nylon polymers (polyamides), which are most typically prepared by condensing polyamines with diacids. Different chain lengths of the constituent polyamines and diacids impart different physical properties to the polymer. These and other bulk chemicals are of special interest to produce from renewable feedstocks via microbial conversion, using either existing or introduced biochemical pathways for producing the chemicals (Chung *et al.*, 2015, Chae *et al.*, 2015; Qian, 2009; Qian 2011).

To develop economically attractive processes for production of bulk chemicals from renewable plant-based carbon feedstocks, three features are essential: high product yields, high productivity, and high product titers. The latter property is particularly important in order to minimize capital equipment and downstream separations costs for product purification. Titers of bulk chemicals in economical fermentation processes often exceed 100 g/L; however, most chemicals at these concentrations (or much lower) exhibit significant toxicity that further reduce yields and productivities by negatively affecting microbial growth.

Escherichia coli being a suitable host for industrial applications, there has been much interest in developing *E. coli* strains with improved tolerance to chemicals of interest for production, such as, *e.g.*, n-butanol, ethanol and isobutanol, or to stress conditions present during fermentation (see, *e.g.*, Sandberg *et al.*, 2014; Lennen and Herrgård, 2014; Tenaillon *et al.*, 2012; Minty *et al.*, 2011; Dragosits *et al.*, 2013; Winkler *et al.*, 2014; Wu *et al.*, 2014; LaCroix *et al.*, 2015; Jensen *et al.*, 2015; Doukyu *et al.*, 2012; Shenhar *et al.*, 2012; and Rath and Jawali, 2006).

Despite these and other advances in the art, there is still a need for bacterial cells with improved tolerance to chemicals of interest for bio-based production, such as polyamines.

SUMMARY OF THE INVENTION

It has been found by the present inventors that certain genetic modifications unexpectedly improve the tolerance of bacterial cells, such as those of the *Escherichia* and *Corynebacterium* genera, to certain chemical compounds, particularly aliphatic polyamines.

Accordingly, the invention provides bacterial cells with improved tolerance to at least one aliphatic polyamine, as well as bacterial cells which are capable of producing an aliphatic polyamine which has improved tolerance to the aliphatic polyamine. Particularly contemplated are putrescine, hexamethylenediamine (HMDA), cadaverine, spermidine, agmatine, 1,3-diaminopropane, ethylenediamine, citrulline, and ornithine.

Also provided are compositions comprising such bacterial cells and an aliphatic polyamine, methods of preparing or screening for such bacterial cells, and methods of producing aliphatic polyamines using such bacterial cells.

These and other aspects and embodiments are described further below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Phase contrast microscope images of putrescine and HMDA evolved isolates containing cell wall or cell shape related mutations (A, top), or or MAGE-reconstructed mutants (B, bottom). Cultures were grown to exponential phase in M9 medium.

Figure 2: Normalized tOD1(evolved)/tOD1(wild-type) for putrescine-evolved isolates grown in the presence of inhibitory concentrations of 12 different chemicals.

Figure 3: Normalized tOD1(evolved)/tOD1(wild-type) for HMDA-evolved isolates grown in the presence of inhibitory concentrations of 12 different chemicals.

DETAILED DISCLOSURE OF THE INVENTION

Accordingly, various aspects of the invention provide for genetically modified bacterial host cells with a higher tolerance to one or more aliphatic polyamines. When transformed with a recombinant biosynthetic pathway for producing the polyamine from a carbon source, the genetically modified bacterial host cells of the invention result in improved production of the polyamine from carbon feedstock, since they maintain robust metabolic activity in the presence of higher concentrations of the polyamine than the unmodified parent cells.

So, in one aspect the bacterial cell comprises a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces expression of an endogenous gene selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, ybeX* and *mpl*, or a combination of any thereof. The bacterial cell may, for example, comprise a genetic modification which reduces expression of *ybeX, proV, cspC, ptsP, wbbK, mpl* or *rph*. Preferably, the genetic modification comprises a knock-down or knock-out of the endogenous gene. In one embodiment, the genetic modification is a knock-out. Optionally, the bacterial cell further comprises a mutation in at least one of YgaC, RpsG, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, MurA, RpsA, SpoT and *argG*.

In one aspect, the bacterial cell comprises genetic modifications which reduce the expression of at least two endogenous genes selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, yicC, yjcF, iscR, yedP, ybeX* and *mpl*. In one embodiment, the bacterial cell comprises genetic modifications which reduce the expression of at least two endogenous genes selected from the group consisting of proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, ybeX and mpl.The bacterial cell may, for example, comprise genetic modifications which reduce the expression of *proV* and at least one of *ptsP, wbbK, cspC* and *yobF.* Preferably, the genetic modification comprises a knock-down or knock-out of the endogenous gene. In one embodiment, the genetic modification is a knock-out. In one embodiment, the genetic modification is a knock-out. Optionally, the bacterial cell further comprises a mutation in at least one of YgaC, RpsG, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, MurA, RpsA, SpoT and *argG*.

In one aspect, the bacterial cell comprises at least one mutated endogenous protein selected from YgaC-R43L, RpsG-L157*, MreB-A298V, MreB-N34K, MreB-E212A, MreB-I24M, MreB-H93N, NusA-L152R, NusA-M204R, SspA-F83C, SspA-V91F, MrdB-E254K, RpoD-E575A, RpoC-V401G, RpoC-V453I, RpoC-R1140C, RpoC-L120P, RpoB-R637L, RpoB-G181V, MurA-G141A, MurA-Y393S, RpsA-D160V, RpsA-D310Y, RpsA-D310G, RpsA-N313K, RpsA-N315K, RpsA-E427R, SpoT-R209H, SpoT-R467H, SpoT-R467L, SpoT-R471H, SpoT-R488C, SpoT-G530C or a C324A mutation in the endogenous gene *argG*.

In one embodiment of any one of the preceding aspects, the bacterial cell may, for example, comprise a genetic modification which reduces expression of *proV* or *ybeX* and at least one mutation or combination of mutations selected from

(i) YgaC-R43L;

(ii) RpsG-L157*;

(iii) RpsG-L157* and MreB-A298V;
(iv) NusA-L152R and SspA-F83C;
(v) MrdB-E254K;
(vi) RpoD-E575A and RpoC-V401G;
(vii) RpoD-E575A, RpoB-R637L, and MurA-Y393S;
(viii) RpsA-D310Y, NusA-M204R, MreB-H93N, and SpoT-R467H; and
(ix) a mutation in *rph* or the *pyrE/rph* intergenic region which increases the expression of *pyrE.*

In a further embodiment of any one of the preceding aspects and embodiments, the genetic modification preferably provides for an increased growth rate, a reduced lag time, or both, of the cell in at least one of putrescine, hexamethylenediamine (HMDA), spermidine, agmatine, 1,3-diaminopropane, cadaverine, ethylenediamine, citrulline, and ornithine.

In a further embodiment of any one of the preceding aspects and embodiments, the bacterial cell comprises a recombinant biosynthetic pathway for producing at least one of putrescine, HMDA, spermidine, agmatine, 1,3-diaminopropane, cadaverine, ethylenediamine, citrulline and ornithine.

In a further embodiment of any one of the preceding aspects and embodiments, the bacterial cell is of the *Escherichia* or *Corynebacterium* genus. Preferably, the bacterial cell is of the *Escherichia coli* species.

In one aspect, there is provided a process for preparing a recombinant bacterial cell, optionally an *E. coli* cell, for producing a polyamine, comprising genetically modifying the cell to

(i) introduce a recombinant biosynthetic pathway for producing a polyamine;

(ii) knock-down or knock-out at least one endogenous gene selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, yicC, yjcF, iscR, yedP, ybeX* and *mpl,* and/or

(iii) introduce at least one mutation selected from YgaC-R43L, RpsG-L157*, MreB-A298V, MreB-N34K, MreB-E212A, MreB-I24M, MreB-H93N, NusA-L152R, NusA-M204R, SspA-F83C, SspA-V91F, MrdB-E254K, RpoD-E575A, RpoC-V401G, RpoC-V453I, RpoC-R1140C, RpoC-L120P, RpoB-R637L, RpoB-G181V, MurA-G141A, MurA-Y393S, RpsA-D160V, RpsA-D310Y, RpsA-D310G, RpsA-N313K, RpsA-N315K, RpsA-E427R, SpoT-

R209H, SpoT-R467H, SpoT-R467L, SpoT-R471H, SpoT-R488C, SpoT-G530C and *argG*-C324A.

In one aspect, there is provided a process for improving the tolerance of an *E. coli* cell to at least one aliphatic polyamine selected from putrescine, HMDA, spermidine, agmatine, 1,3-diaminopropane, cadaverine, ethylenediamine, citrulline and ornithine, comprising

(i) genetically modifying the cell to knock-down or knock-out at least one endogenous gene selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, yicC, yjcF, iscR, yedP, ybeX* and *mpl*;

(ii) preparing a population of *E. coli* cells comprising one or more mutations in at least one endogenous gene selected from *ygaC*, *rpsG*, *mreB*, *nusA*, *sspA*, *mrdB, rpoD, rpoC, rpoB, murA,rpsA, spoT* and *argG*; and selecting any host cell which has an improved tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, agmatine, ethylenediamine, citrulline and ornithine; or

(iii) both i) and ii).

In one aspect, there is provided a method for producing an aliphatic polyamine, comprising culturing the bacterial cell of any one of the preceding aspects or embodiments in the presence of a carbon source.

In one aspect, there is provided a composition comprising putrescine, HMDA, spermidine, agmatine, cadaverine, 1,3-diaminopropane, ethylenediamine, citrulline, or ornithine at a concentration of at least 10 g/L, such as at least 25 g/L g/L, and a plurality of bacterial cells of the *Escherichia* genus which comprise

(i) at least one genetic modification which reduces expression of an endogenous gene selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, yicC, yjcF, iscR, yedP, ybeX* and *mpl*, or a combination of any thereof;

(ii) a mutation in at least one of *ygaC*, *rpsG*, *mreB*, *nusA*, *sspA*, *mrdB, rpoD, rpoC, rpoB, murA, rpsA, spoT* and *argG* which improves the tolerance of the bacterial cell to putrescine, HMDA, spermidine, agmatine, cadaverine, 1,3-diaminopropane, ethylenediamine, citrulline, or ornithine; or

(iii) a combination of a) and b).

Definitions

An "aliphatic polyamine" as used herein is an organic compound comprising an aliphatic carbon chain to which two or more primary amino (-NH_2) groups are attached, and includes linear aliphatic polyamines and derivatives thereof. Aliphatic polyamines suitable for production in bacteria typically comprise from 2 to 12 carbon atoms, preferably 2 to 10 carbon atoms, more preferably 2 to 8 carbon atoms, and, most preferably, 2 to 6 carbon atoms, and, optionally comprises one or more heteroatoms such as, *e.g.*, O, N or S. Linear aliphatic polyamines comprising 2, 3 or 4 primary amino groups are preferred and include, but are not limited to, ethylenediamine (1,2-diaminoethane), 1,3-diaminopropane (propane-1,3-diamine), putrescine (butane-1,4-diamine), cadaverine (pentane-1,5-diamine), spermidine (*N*-(3-aminopropyl)-1,4-diaminobutane, agmatine (1-amino-4-guanidinobutane), spermine (N,N'-bis(3-aminopropyl)-1,4-diaminobutane) and hexamethylenediamine (hexane-1,6-diamine; HMDA), as well as amino acids containing multiple amines, such as, *e.g.*, citrulline, ornithine, carnitine, 2,6-diaminopimelic acid, arginine and lysine. Linear aliphatic diamines having, *e.g.*, 2 to 8 carbon atoms and which do not contain any heteroatoms other than nitrogen (N), such as, *e.g.*, putrescine, HMDA, 1,3-diaminopropane, ethylenediamine, spermidine and cadaverine, and amino acids containing multiple amines, such as, *e.g.*, citrulline and ornithine, are most preferred.

As used herein, a "recombinant biosynthetic pathway" for a compound of interest refers to an enzymatic pathway resulting in the production of a compound of interest in a host cell, wherein at least one of the enzymes is expressed from a transgene, *i.e.*, a gene added to the host cell genome by transformation. In some cases, the recombinant biosynthetic pathway also comprises a deletion of one or more native genes in the host cell. The compound of interest is typically a polyamine, such as an aliphatic polyamine, and may be the actual end product or a precursor or intermediate in the production of another end product.

The terms "tolerant" or "improved tolerance", when used to describe a genetically modified bacterial cell of the invention or a strain derived therefrom, refers to a genetically modified bacterial cell or strain that shows a reduced lag time, an improved growth rate, or both, in the presence of an aliphatic polyamine than the parent bacterial cell or strain from which it is derived, typically at concentrations of at least 5 g/L, such as at least 10 g/L, such as at least 15 g/L, such as at least 19 g/L, such as at least 20 g/L, such as at least 25 g/L, such as at least 30 g/L, such as at least 35 g/L, such as at least 38 g/L, such as at least 40 g/L. An improved growth rate is at least 5%, such as at least 10%, such as at least 20%, such as at least 50%, such as at least 75% higher than that of a control, typically the parent cell or strain. A reduced lag time is at least 10%, such as at least 20%, such as at least 50%, such

as at least 75%, such as at least 90% shorter than that of a control, typically the parent cell or strain.

The term "gene" refers to a nucleic acid sequence that encodes a cellular function, such as a protein, optionally including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. An "endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "transgene" is a gene, native or heterologous, that has been introduced into the genome by a transformation procedure.

As used herein the term "coding sequence" refers to a DNA sequence that encodes a specific amino acid sequence.

The term "native", when used to characterize a gene or a protein herein with respect to a host cell, refers to a gene or protein having the nucleic acid or amino acid sequence as found in the host cell.

The term "heterologous", when used to characterize a gene or protein with respect to a host cell, refers to a gene or protein which has a nucleic acid or amino acid sequence not normally found in the host cell.

As used herein the term "transformation" refers to the transfer of a nucleic acid fragment, such as a gene, into a host cell. Host cells containing a gene introduced by transformation or a "transgene" are referred to as "transgenic" or "recombinant" or "transformed" cells.

As used herein, a "genetic modification" refers to the introduction a genetically inherited change in the host cell genome. Examples of changes include mutations in genes and regulatory sequences, mutations in coding and non-coding DNA sequences. "Mutations" include deletions, substitutions and insertion of nucleic acids or nucleic acid fragments in the genome.

The term "expression", as used herein, refers to the process in which a gene is transcribed into mRNA, and may optionally include the subsequent translation of the mRNA into an amino acid sequence, *i.e.*, a protein or polypeptide.

As used herein, "reduced expression" or "downregulation" of an endogenous gene in a host cell means that the levels of the mRNA, protein and/or protein activity encoded by the gene are significantly reduced in the host cell, typically by at least 25%, such as at least 50%,

such as at least 75%, such as at least 90%, such as at least 95%, as compared to a control. Typically, when the reduced expression is obtained by a genetic modification in the host cell, the control is the unmodified host cell. Sometimes, *e.g.*, in the case of gene knock-out, the reduction of native mRNA and functional protein encoded by the gene is higher, such as 99% or greater.

"Increased expression", "upregulation", "overexpressing" or the like, when used in the context of a protein or activity described herein, means increasing the protein level or activity within a bacterial cell. An up-regulation of an activity can occur through, *e.g.*, increased activity of a protein, increased potency of a protein or increased expression of a protein. The protein with increased activity, potency or expression can be encoded by genes disclosed herein.

Genetic modifications resulting in a reduced expression of a target gene/protein can include, *e.g.*, knock-down of the gene (*e.g.*, a mutation in a promoter that results in decreased gene expression), a knock-out of the gene (*e.g.*, a mutation or deletion of the gene that results in 99 percent or greater decrease in gene expression), a mutation or deletion in the coding sequence which results in the expression of non-functional protein, and/or the introduction of a nucleic acid sequence that reduces the expression of the target gene, *e.g.* a repressor that inhibits expression of the target or inhibitory nucleic acids (*e.g.* CRISPR etc.) that reduces the expression of the target gene.

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, 4th ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 2012; and by Silhavy, T. J., Bennan, M. L. and Enquist, L. W. Experiments with Gene Fusions; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1984; and by Ausubel, F. M. *et al.*, In Current Protocols in Molecular Biology, published by John Wiley & Sons (1995); and by Datsenko and Wanner, 2000; and by Baba *et al.*, 2006; and by Thomason *et al.*, 2007.

A "conservative" amino acid substitution in a protein is one that does not negatively influence protein activity. Typically, a conservative substitution can be made within groups of amino acids sharing physicochemical properties, such as, *e.g.*, basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagines), hydrophobic amino acids (leucine, isoleucine, valine and methionine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, and threonine). Most commonly, substitutions can be

made between Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly. Other preferred substitutions are set out in Table 1 below. The following list shows exampes of amino acid substitutions:

Original amino acid	Examples of substitutions	Preferable substitution
Ala (A)	val; leu; ile	Val
Arg (R)	lys; gln; asn	Lys
Asn (N)	gln; his; asp, lys; arg	Gln
Asp (D)	glu; asn	Glu
Cys (C)	ser; ala	Ser
Gln (Q)	asn; glu	Asn
Glu (E)	asp; gln	Asp
Gly (G)	Ala	Ala
His (H)	asn; gln; lys; arg	Arg
Ile (I)	leu; val; met; ala; phe; norleucine	Leu
Leu (L)	norleucine; ile ; val; met; ala; phe	Ile
Lys (K)	arg; gln; asn	Arg
Met (M)	leu; phe; ile	Leu
Phe (F)	leu; val; ile; ala; tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	thr	Thr
Thr (T)	Ser	Ser
Trp (W)	tyr; phe	Tyr
Tyr (Y)	trp; phe ; thr; ser	Phe
Val (V)	ile; leu; met; phe; ala; norleucine	Leu

In the numbers in the Tables and Figures 3 and 4, a comma represents the decimal mark.

Specific embodiments of the invention

As described in the Examples, the maximum measured concentrations of putrescine and HMDA at which native K-12 MG1655 strain could grow was 40 g/L, respectively, with zero growth detected at 40 g/L and 50 g/L concentrations of putrescine and HMDA, respectively, thus limiting the economic feasibility of production of aliphatic polyamines as platform chemicals. By contrast, bacterial cells comprising one or more mutations according to the invention exhibit a dramatically improved growth at high concentrations of aliphatic

polyamines such as putrescine and/or HMDA, *e.g.*, concentrations of 10 g/L or more, such as 25 g/L or more, typically reflected by an increased growth rate, a reduced lag time, or both.

So, provided are bacterial cells with improved tolerance to at least one aliphatic polyamine, such as one or more of putrescine, HMDA, cadaverine, 1,3-diaminopropane, ethylenediamine, spermidine and cadaverine, and amino acids containing multiple amines, such as, *e.g.*, citrulline and ornithine, as well as related processes and materials for producing and using such bacterial cells.

1) Genetic modifications

The genetic modifications according to the invention include those resulting in reduced expression of genes, *e.g.*, by gene knock-down or knock-out, herein referred to as "Group 1 modifications"; as well as silent mutations in coding or non-coding regions and non-silent (*i.e.*, coding) mutations in coding regions, herein referred to as "Group 2 modifications"; and combinations thereof, as described below.

a) Group 1 modifications

In one aspect, the bacterial cell has a genetic modification which reduces the expression of one or more endogenous genes selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, ybeX* and *mpl*. For example, in one particular embodiment, the one or more endogenous genes are selected from *ybeX, proV, cspC, ptsP, wbbK, mpl* and *rph*. In one embodiment, the endogenous gene is selected from *ybeX, proV, cspC, ptsP, wbbK, mpl* or *rph*.

In one aspect, there is provided a bacterial cell with improved tolerance to at least one of putrescine, 1,3-diaminopropane, and cadaverine, such as, *e.g.*, to putrescine, comprising a genetic modification which reduces the expression of one or more endogenous genes selected from *proV, proW, proX, cspC, ptsP*, *rph* and *mpl.* In one embodiment, the endogenous gene is selected from one or more of *proV, cspC, ptsP, rph* and *mpl*. In one embodiment, the bacterial cell comprises a genetic modification in, *e.g.*, a knock-out or deletion of *proV, cspC,* or *rph.* In one embodiment, the bacterial cell comprises a knock-out or deletion of *proV; cspC*; *proV* and *cspC*; *proV* and *ptsP*; *proV*, *ptsP* and *wbbK*; *proV, ptsP* and *mpl*; or of *proV, cspC*, and *mpl.* In another embodiment, the bacterial cell comprises a knock-out, *e.g.*, a deletion, of *proV* and at least one of *ptsP, cspC, and mpl*; *proV, ptsP,* and *mpl;* and *proV, cspC,* and *mpl.*

In one aspect, there is provided a bacterial cell with improved tolerance to at least one of HMDA, spermidine, citrulline, and ornithine, such as, *e.g.*, to HMDA, comprising a genetic modification which reduces the expression of one or more endogenous genes selected from *proV, proW, proX, ptsP, wbbK, ybeX, mpl* and *rph.* In one embodiment, the endogenous gene is selected from one or more of *proV, ptsP, wbbK, ybeX, mpl* and *rph*. In one embodiment, the bacterial cell comprises a genetic modification in, *e.g.*, a knock-out or deletion of, *proV, ptsP, wbbK, ybeX, mpl* or *rph.* In one embodiment, the bacterial cell comprises a knock-out, *e.g.*, a deletion, of *proV; ptsP; wbbK; ybeX; mpl; rph;*or *proV* and *ptsP*, optionally in combination with one or more of *wbbK* and *nagC*. In another embodiment, the bacterial cell comprises a knock-out, *e.g.,* a deletion, of *ybeX* and *mpl*; *proV*, *ptsP* and *ybeX*; *proV*, *ptsP*, *ybeX* and *mpl*; *proV, cspC* and *mpl*; *proV, cspC and ybeX*; or of *proV, cspC, mpl* and *ybeX*. In another embodiment, the bacterial cell comprises a knock-out or deletion of *proV* and at least one of *ptsP, cspC, mpl*, and *ybeX*; *proV, ptsP,* and at least one of *mpl* and *ybeX; proV, cspC,* and at least one of *mpl* and *ybeX;ybeX* and at least one of *proV, ptsP, cspC,* and *mpl; proV, ptsP, ybeX,* and *mpl;* and*proV, cspC, ybeX,* and *mpl.*

In one aspect, there is provided a bacterial cell which comprises genetic modifications reducing the expression of at least two endogenous genes selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, rph, ybeX* and *mpl*. In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of a gene selected from *ybeX, proV, cspC, ptsP, wbbK, mpl* and *rph*. In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of *proV* and a second genetic modification which reduces the expression of a gene selected from *cspC, ptsP, wbbK, yobF, nagC, nagA, rph,ybeX* and *mpl.* In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of *proW* and a second genetic modification which reduces the expression of a gene selected from *proV, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, ybeX* and *mpl*. In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of *proX* and a second genetic modification which reduces the expression of a gene selected from *proV, proW, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, ybeX* and *mpl*. In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of *cspC* and a second genetic modification which reduces the expression of a gene selected from *proV, proW, proX, ptsP, wbbK, yobF, nagC, nagA, rph, ybeX* and *mpl*. In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of *ptsP* and a second genetic modification which reduces the expression of a gene selected from *proV, proW, proX, cspC, wbbK, yobF, nagC, nagA, rph, ybeX* and *mpl*. In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of *wbbK* and a second genetic modification which reduces the expression of a gene selected from *proV, proW, proX,*

cspC, ptsP, yobF, nagC, nagA, rph, ybeX and *mpl*. In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of *yobF* and a second genetic modification which reduces the expression of a gene selected from *proV, proW, proX, cspC, ptsP, wbbK, nagC, nagA, rph, ybeX* and *mpl*. In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of *nagC* and a second genetic modification which reduces the expression of a gene selected from *cspC, ptsP, wbbK, yobF, rph, ybeX* and *mpl*. In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of *nagA* and a second genetic modification which reduces the expression of a gene selected from *cspC, ptsP, wbbK, yobF, nagC, rph, ybeX* and *mpl*. In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of *rph* and a second genetic modification which reduces the expression of a gene selected from *cspC, ptsP, wbbK, yobF, nagC, nagA, ybeX* and *mpl*. In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of *ybeX* and a second genetic modification which reduces the expression of a gene selected from *cspC, ptsP, wbbK, yobF, nagC, nagA, rph* and *mpl*. In one embodiment, the bacterial cell comprises a first genetic modification which reduces the expression of *mpl* and a second genetic modification which reduces the expression of a gene selected from *cspC, ptsP, wbbK, yobF, nagC, nagA, rph* and *ybeX*. In one specific embodiment, either one or both of the first and second genetic modifications is a knock-out of the gene, optionally a deletion. In an alternative embodiment at least one of the first and second genetic modifications is a knock-down of the gene.

In one aspect, there is provided a bacterial cell according to any one of the preceding aspects and embodiments, wherein the genetic modification is a knock-down of the one or more endogenous genes, resulting in at least 25%, such as at least 50%, such as at least 75%, such as at least 90%, such as at least 95%, reduction in the level of mRNA encoded by the gene.

In one aspect, there is provided a bacterial cell according to any one of the preceding aspects and embodiments, wherein the genetic modification is a knock-down of the one or more endogenous genes, resulting in at least 25%, such as at least 50%, such as at least 75%, such as at least 90%, such as at least 95%, reduction in the level of protein encoded by the gene.

In one aspect, there is provided a bacterial cell according to any one of the preceding aspects and embodiments, wherein the genetic modification is a knock-out of the one or more endogenous genes.

Knock-down or knock-out of a gene can be accomplished by any method known in the art for bacterial cells, and include, *e.g.*, lambda Red mediated recombination, P1 phage transduction, and single-stranded oligonucleotide recombineering/MAGE technologies (see, *e.g.*, Datsenko and Wanner, 2000; Thomason *et al.*, 2007; Wang *et al.*, 2009). Typically, a knock-down of a gene can be accomplished by, for example, a mutation in the promoter region resulting in decreased transcription, a deletion or mutation in the coding region of the gene resulting in a reduced activity of the protein, or by the presence of antisense sequences that interfere with transcription or translation of the gene, resulting in reduced expression of the protein. Preferably, the knocking-down of a gene results in at least 20% reduction in the expression level of the gene product in the bacterial cell, such as at least 30%, such as at least 40%, such as at least 50%, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 95% or higher.

A knock-out of a gene includes elimination of a gene's expression, such as by introducing a mutation in the coding sequence and/or promoter so that at least a portion (up to and including all) of the coding sequence and/or promoter is disrupted or deleted deletion, mutation, or insertion, resulting in loss of expression of the protein, or expression only of a non-functional mutant or non-functional fragment of the endogenous protein. As used herein, the symbol "DELTA" denotes a deletion of an endogenous gene. Preferably, a knock-out of a gene results in 1% or less of the gene product being detectable, such as no detectable gene product.

In one aspect, the bacterial cell of any aspect or embodiment described herein comprises a mutation in at least one of YgaC, RpsG, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, MurA, RpsA, SpoT and *argG* which provides for improved tolerance to at least one aliphatic polyamine, such as one or more of putrescine, HMDA, cadaverine, 1,3-diaminopropane, spermidine, agmatine, ethylenediamine, citrulline, and ornithine. The mutated protein can be expressed from a mutated version of the endogenous gene, or from a transgene. Advantageously, these mutations can be combined with each other and/or with one or more modifications described in the preceding sections.

b) Group 2 modifications

In one embodiment, the bacterial cell comprises a mutation in YgaC which increases tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline and ornithine. In one particular embodiment, the YgaC comprises a mutation, such as a deletion or amino acid substitution, in residue R43. Preferably, the mutation is R43L or a conservative amino acid substitution thereof. In one particular embodiment, the bacterial cell further comprises at least one Group 1 modification, an

additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW*, *proX*, *ybeX*, or *mpl*, such as *proV*, and the Group 2 modification a mutation in one or more of RpsG, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, MurA, RpsA, SpoT and *argG*. In one embodiment, where the bacterial cell comprises a Group 1 modification which reduces the expression of *ybeX*, the alipahtic diamine is not putrescine, 1,3-diaminopropane, or ethylenediamine.

In one embodiment, the bacterial cell comprises a mutation in RpsG which increases tolerance to putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline, ornithine, or any combination thereof. In one particular embodiment, the RpsG comprises a mutation, such as a coding mutation or amino acid substitution, in residue L157 or W156. Preferably, the mutation is a coding mutation that introduces a translation stop codon. In one particular embodiment, the bacterial cell further comprises at least one Group 1 modification, at least one additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW proX*, *ybeX,* or *mpl*, such as *proV*, and the Group 2 modification a mutation in one or more of YgaC, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, MurA, RpsA, SpoT and *argG*, such as *argG* or MreB. In one particular embodiment, the additional Group 2 modifications do not consist of only an R471H mutation in SpoT.

In one embodiment, the bacterial cell comprises a mutation in MreB which increases tolerance to putrescine, HMDA, 1,3-diaminopropane, cadaverine, ethylenediamine, ornithine or combinations thereof. In one particular embodiment, the MreB comprises a mutation, such as a deletion or amino acid substitution, in residue A298. Preferably, the mutation is an A298V, N34K, E212A, I24M, or H93N substitution or a conservative substitution thereof. In one particular embodiment, the bacterial cell further comprises at least one Group 1 modification, at least one additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW, proX, ybeX*, or *mpl* , such as *proV*, and the Group 2 modification can be a mutation in one or more of YgaC, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, MurA, RpsA, SpoT and *argG*, such as RpsG.

In one embodiment, the bacterial cell comprises a mutation in NusA or NusG which increases tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, citrulline and ornithine. In one particular embodiment, the NusA comprises a mutation, such as a deletion or amino acid substitution, in residue L152. Preferably, the mutation is an

L152R or M204R substitution or a conservative substitution thereof. In one particular embodiment, the NusG comprises a mutation, such as a deletion or amino acid substitution, in residue G166, such as G166V. In one embodiment, the bacterial cell further comprises an additional Group 2 modification, such as a mutation SspA, such as SspA-F83C or a conservative substitution thereof. In another particular embodiment, the bacterial cell further comprises at least one Group 1 modification, at least one additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW, proX*, *ybeX,* or *mpl*, such as *proV*, and the Group 2 modification can be a mutation in one or more of YgaC, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, MurA, RpsA, SpoT and *argG*, such as SspA.

In one embodiment, the bacterial cell comprises a mutation in SspA which increases tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline and ornithine. In one particular embodiment, the SspA comprises a mutation, such as a deletion or amino acid substitution, in residue F83 or V91. Preferably, the mutation is an F83C or V91F substitution or a conservative substitution thereof. In one embodiment, the bacterial cell further comprises an additional Group 2 modification, such as a mutation NusA, such as NusA-L152R or NusA-M204R, or conservative substitutions thereof. In another particular embodiment, the bacterial cell further comprises at least one Group 1 modification, at least one additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW*,*proX*, *ybeX,* or *mpl*, such as *proV*, and the Group 2 modification can be a mutation in one or more of YgaC, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, MurA, RpsA, SpoT and *argG*, such as NusA.

In one embodiment, the bacterial cell comprises a non-coding mutation in *argG* which increases tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline and ornithine, for example a cytidine (C) to adenosine (A) substitution in position 324 of the nucleic acid sequence, *i.e.*, in the codon corresponding to amino acid residue A108. In one particular embodiment, the bacterial cell further comprises at least one Group 1 modification, at least one additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW*, *proX*, *ybeX,* or *mpl,* such as *proV*, and the Group 2 modification can be a mutation in one or more of YgaC, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, MurA, RpsA, SpoT and *argG*, such as RpsG.

In one embodiment, the bacterial cell comprises a mutation in MrdB which increases tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline, ornithine. In one particular embodiment, the MrdB comprises a mutation, such as a deletion or amino acid substitution, in residue E254. Preferably, the mutation is an E254K substitution or a conservative substitution thereof. In one embodiment, the bacterial cell further comprises an additional Group 2 modification, such as a mutation in RpoB or RpsA, such as RpoB-R637L or RpsA-D160V, or conservative substitutions thereof. In another particular embodiment, the bacterial cell further comprises at least one Group 1 modification, at least one additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW*,*proX*, *ybeX,* or *mpl*, such as *proV*, and the Group 2 modification can be a mutation in one or more of YgaC, MreB, NusA, SspA, RpoD, RpoC, RpoB, MurA, RpsA, SpoT and *argG*, such as RpoB.

In one embodiment, the bacterial cell comprises a mutation in RpoD which increases tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline and ornithine. In one particular embodiment, the RpoD comprises a mutation, such as a deletion or amino acid substitution, in residue E575. Preferably, the mutation is an E575A substitution or a conservative substitution thereof. In one embodiment, the bacterial cell further comprises an additional Group 2 modification, such as a mutation in RpoC, such as RpoC-V401G, or conservative substitutions thereof. In another particular embodiment, the bacterial cell further comprises at least one Group 1 modification, at least one additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW*,*proX*, *ybeX,* or *mpl*, such as *proV*, and the Group 2 modification can be a mutation in one or more of YgaC, MreB, NusA, SspA, MrdB, RpoC, RpoB, MurA, RpsA, SpoT and *argG*, such as RpoC.

In one embodiment, the bacterial cell comprises a mutation in RpoC which increases tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline and ornithine. In one particular embodiment, the RpoC comprises a mutation, such as a deletion or amino acid substitution, in residue V401, V453, R1140, or L120. Preferably, the mutation is a V401G, V453I, R1140C, or L120P substitution or a conservative substitution thereof. In one embodiment, the bacterial cell further comprises an additional Group 2 modification, such as a mutation in RpoC, such as RpoD-E575A, or conservative substitutions thereof. In another particular embodiment, the bacterial cell further comprises at least one Group 1 modification, at least one additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the

Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW*,*proX*, *ybeX,* or *mpl*, such as *proV*, and the Group 2 modification can be a mutation in one or more of YgaC, MreB, NusA, SspA, MrdB, RpoD, RpoB, MurA, RpsA, SpoT and *argG*, such as RpoD.

In one embodiment, the bacterial cell comprises a mutation in RpoB which increases tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline and ornithine. In one particular embodiment, the RpoB comprises a mutation, such as a deletion or amino acid substitution, in residue R637 or G181. Preferably, the mutation is an R637L or G181V substitution or a conservative substitution thereof. In one embodiment, the bacterial cell further comprises an additional Group 2 modification, such as a mutation in RpoC, such as MurA-Y393S, or conservative substitutions thereof. In another particular embodiment, the bacterial cell further comprises at least one Group 1 modification, at least one additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW*,*proX*, *ybeX,* or *mpl*, such as *proV*, and the Group 2 modification can be a mutation in one or more of YgaC, MreB, NusA, SspA, MrdB, RpoD, RpoC, MurA, RpsA, SpoT and *argG*, such as MurA.

In one embodiment, the bacterial cell comprises a mutation in MurA which increases tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline and ornithine. In one particular embodiment, the MurA comprises a mutation, such as a deletion or amino acid substitution, in residue G141 or Y393. Preferably, the mutation is an G141A or Y393S substitution or a conservative substitution thereof. In one embodiment, the bacterial cell further comprises an additional Group 2 modification, such as a mutation in RpoD, such as RpoD-E575A, or conservative substitutions thereof. In another particular embodiment, the bacterial cell further comprises at least one Group 1 modification, at least one additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW*,*proX*, *ybeX,* or *mpl*, such as *proV*, and the Group 2 modification can be a mutation in one or more of YgaC, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, RpsA, SpoT and *argG*, such as RpoD.

In one embodiment, the bacterial cell comprises a mutation in RpsA which increases tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline and ornithine. In one particular embodiment, the RpsA comprises a mutation, such as a deletion or amino acid substitution, in residue D160, D310, N313, N315, or E427. Preferably, the mutation is a D160V, D310Y, D310G, N313K, N315K, or

E427R substitution or a conservative substitution thereof. In one embodiment, the bacterial cell further comprises an additional Group 2 modification, such as a mutation in NusA, such as NusA-M204R, or conservative substitutions thereof. In another particular embodiment, the bacterial cell further comprises at least one Group 1 modification, at least one additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW*,*proX*, *ybeX,* or *mpl*, such as *proV*, and the Group 2 modification can be a mutation in one or more of YgaC, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, MurA, SpoT and *argG*, such as NusA.

In one embodiment, the bacterial cell comprises a mutation in SpoT which increases tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline and ornithine. In one particular embodiment, the SpoT comprises a mutation, such as a deletion or amino acid substitution, in residue R209, R467, R471, R488 or G530. Preferably, the mutation is a R209H, R467H, R467L, R471H, R488C, or G530C substitution or a conservative substitution thereof. In one embodiment, the bacterial cell further comprises an additional Group 2 modification, such as a mutation in MreB, such as MreB-E212A, MreB-I24M, MreB-H93N, MreB-A298V, or conservative substitutions thereof. In another particular embodiment, the bacterial cell further comprises at least one Group 1 modification, at least one additional Group 2 modification, or both, according to any aspects or embodiments herein. For example, the Group 1 modification can be a genetic modification which reduces the expression of *proV*, *proW*,*proX*, *ybeX,* or *mpl*, such as *proV*, and the Group 2 modification can be a mutation in one or more of YgaC, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, MurA, RpsA and *argG*, such as MreB. In one particular embodiment, the additional Group 2 modifications do not consist of only an L157* or W156* mutation in RpsG. In another particular embodiment, the bacterial cell comprises a mutation in SpoT and one or more further genetic modifications..

In separate and specific embodiments, the bacterial cell comprises

a) a genetic modification which reduces expression of *proV,* and at least one mutation selected from RpsG-L157* and MreB-A298V, optionally wherein the aliphatic polyamine is selected from putrescine, 1,3-diaminopropane, cadaverine, spermidine, citrulline and ornithine;

b) a genetic modification which reduces expression of *proV,* and mutations RpsG-L157* and MreB-A298V, optionally wherein the aliphatic polyamine is selected from putrescine, 1,3-diaminopropane, cadaverine, spermidine, citrulline and ornithine;

c) a genetic modification which reduces expression of *proV,* and a mutation YgaC-R43L, optionally wherein the aliphatic polyamine is selected from putrescine, 1,3-diaminopropane, cadaverine, spermidine, citrulline and ornithine;

d) a genetic modification which reduces expression of *ybeX*, and at least one of *proV, ptsP, cspC,* and *mpl*, and at least one mutation selected from SspA-F83C, NusA-L152R, RpsG-L157*, YgaC-R43L, and MreB-A298V, optionally wherein the aliphatic polyamine is selected from HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline, and ornithine;

e) a genetic modification which reduces expression of *ybeX* and *mpl*, and mutations NusA-L152R and SspA-F83C, optionally wherein the aliphatic polyamine is selected from HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline, and ornithine;

f) a genetic mutation which reduces expression of *ybeX* and *mpl*, and mutations RpsG-L157* and MreB-A298V, optionally wherein the aliphatic polyamine is selected from HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline, and ornithine;

g) a genetic mutation which reduces expression of *ybeX* and *mpl*, and mutation YgaC-R43L, optionally wherein the aliphatic polyamine is selected from HMDA, 1,3-diaminopropane, cadaverine, spermidine, ethylenediamine, citrulline, and ornithine.

In an alternative embodiment, the bacterial cell comprises an upregulation of at least one of YgaC, RpsG, MreB, NusA, SspA, MrdB, RpoD, RpoC, MurA, RpsA, SpoT and *argG, e.g.*, by transforming the bacterial cell with a transgene expressing the endogenous protein or a functionally active variant thereof, *e.g.*, RpsG-L157*, MreB-A298V, MreB-N34K, MreB-E212A, MreB-I24M, MreB-H93N, NusA-L152R, NusA-M204R, SspA-F83C, SspA-V91F, MrdB-E254K, RpoD-E575A, RpoC-V401G, RpoC-V453I, RpoC-R1140C, RpoC-L120P, RpoB-R637L, RpoB-G181V, MurA-G141A, MurA-Y393S, RpsA-D160V, RpsA-D310Y, RpsA-D310G, RpsA-N313K, RpsA-N315K, RpsA-E427R, SpoT-R209H, SpoT-R467H, SpoT-R467L, SpoT-R471H, SpoT-R488C, SpoT-G530C and *argG*-C324A. To cause an up-regulation through increased expression of a protein, the copy number of a gene or genes encoding the protein may be increased. Alternatively, a strong and/or inducible promoter can be used to direct the expression of the gene, the gene being expressed either as a transient expression vehicle or homologously or heterologously incorporated into the bacterial genome. In another embodiment, the promoter, regulatory region and/or the ribosome binding site upstream of

the gene can be altered to achieve the over-expression. The expression can also be enhanced by increasing the relative half-life of the messenger or other forms of RNA. Any one or a combination of these approaches can be used to effect upregulation of a desired target protein as needed.

In one embodiment, the bacterial cell comprises one or more mutations which increase(s) the expression level or activity of PyrE. *E. coli* K-12 MG1655 and W3110, plus their common ancestor strain W1485, are known to exhibit pyrimidine starvation in minimal media due to the presence a frameshift mutation occurring in *rph* relative to other *E. coli* strains (Jensen *et al.*, 1993). This mutation disrupts the transcriptional/translational coupling required for efficient translation of *pyrE*, encoding orotate phosphoribosyltransferase in the pyrimidine biosynthesis pathway. Compensatory mutations that correct this deficiency are well-known in the art. One of these mutations is an 82 bp deletion near the 3' terminus of *rph*, due to presence of two homologous GCAGAAGGC sequences flanking this 82 bp region (Conrad *et al.*, 2009). This mutation precisely corresponds to the 82 bp deletion found in resequenced isolates from populations HMDA4 and HMDA6, and isolates PUTR6-7 and PUTR6-10 (from NC_000913.3 coordinates 3815859 to 3815931; Table 4). In addition to the 82 bp deletion, a 1 bp deletion at coordinate 3815809 in the pyrE/rph intrgenic region has previously been encountered in strains evolved for growth on a minimal glucose medium (LaCroix *et al.*, 2015), and a wide array of other frameshift mutations, substitutions, and coding mutations near the 3' terminus of *rph* were encountered in a short-term selection/evolution of combinatorial mutant libraries in minimal medium at an elevated temperature of 42°C (Sandberg *et al.*,2014). The same 1 bp deletion in the *pyrE/rph* intergenic region was also found to be present in evolved isolates HMDA3-4, HMDA3-5, HMDA3-6, HMDA5-4, HMDA5-5, and HMDA5-10. Another 1 bp deletion in the *pyrE/rph* intergenic region was found at coordinate 3815801 in evolved isolates PUTR8-3, PUTR8-6, PUTR8-10, HMDA2-1, HMDA2-8, HMDA8-5, HMDA8-9, and HMDA8-10. Furthermore, intergenic mutations between *rph* and *yicC* at coordinate 3816611 (C to A mutation) were found in resequenced isolates from population PUTR3 and HMDA1. Without being limited to theory, all of these mutations can serve the same function of increasing expression of PyrE, with the selective pressure for these mutations being even stronger in minimal media with particular imposed stresses (certain chemicals or heat) than in minimal media alone. In one embodiment, the bacterial cell comprises mutations in *rph* or the *pyrE/rph* intergenic region, such as, *e.g.*, an 82 bp deletion near the 3' terminus of *rph*, an intergenic C to A mutation at coordinate 3816611 in the intergenic region between *rph* and *yicC*, or 1 or 82 bp deletions in the intergenic region between *pyrE* and *rph*.

2) Production pathways

In one aspect, there is provided a bacterial cell with improved tolerance to at least one aliphatic polyamine according to any aspect or embodiment described herein, wherein the bacterial cell further comprises a recombinant biosynthetic pathway for producing an aliphatic polyamine of interest, such as, *e.g.*, putrescine, HMDA, spermidine, agmatine, cadaverine, 1,3-diaminopropane, citrulline or ornithine. In principle, any such recombinant biosynthetic pathway which is known in the art can be introduced into the cell by standard recombinant technologies. Some specific, preferred pathways are, however, exemplified below and in Example 1, section l). Preferably, the bacterial cell comprising the recombinant biosynthetic pathway produces at least 2 times, at least three times, at least 5 times or at least 10 times or more of the aliphatic polyamine than the wild-type bacterial cell during a predetermined time period, *e.g.*, 24h or more, under the same conditions, *i.e.*, conditions suitable for producing the aliphaitc polyamine. It is to be understood that, when a specific enzyme of these biosynthetic pathways is mentioned by name such as, *e.g.*, "acetylglutamate kinase", the enzyme may be any characterized and sequenced enzyme, from any species, that have been reported in the literature so long as it provides the desired activity. In some embodiments, the enzyme is an overexpressed gene which is native to the host cell used. In some embodiments, the enzyme is a functionally active fragment or variant of an enzyme which is heterologous or native to the host cell. Also, in some embodiments, the recombinant biosynthetic pathway comprises a knock-down or a knock-out of one or more genes, typically for the purpose of avoiding competing reactions reducing the yield of the desired aliphatic polyamine.

So, in one embodiment, the biosynthetic pathway is for producing putrescine and comprises overexpressed or de-regulated N-acetylglutamate kinase (ArgB; EC 2.7.2.8), N-acetyl-glutamylphosphate reductase (ArgC; EC 1.2.1.38), N-acetylornithine aminotransferase/N-succinyldiaminopimelate aminotransferase (ArgD; EC 2.6.1.11), acetylornithine deacetylase (ArgE; EC 3.5.1.16), putrescine:H^+ symporter/putrescine: ornithine antiporter (PotE), and ornithine decarboxylate (SpeC and/or SpeF; EC 4.1.1.17), and a knock-down or knock-out of any native ornithine carbamoyltransferase (ArgI and/or ArgF; EC 2.1.3.3), spermidine synthase (SpeE; EC 2.5.1.16), spermidine acetyltransferase (SpeG; EC 2.3.1.57), glutamate-putrescine ligase (PuuA; EC 6.3.1.11), putrescine:H^+ symporter (PuuP), and RNA polymerase sigma S (sigma 38) factor (RpoS). In a preferred embodiment, the pathway additionally comprises an overexpressed or de-regulated N-acetylglutamate synthase (ArgA; EC 2.3.1.1). In a preferred embodiment, the bacterial cell is an *E. coli* cell and comprises overexpressed N-acetylglutamate kinase (ArgB; EC 2.7.2.8), N-acetylglutamylphosphate reductase (ArgC; EC 1.2.1.38), N-acetylornithine aminotransferase/N-succinyldiaminopimelate aminotransferase (ArgD; EC 2.6.1.11), acetylornithine deacetylase (ArgE; EC 3.5.1.16),

putrescine:H^+ symporter/putrescine: ornithine antiporter (PotE), and ornithine decarboxylate (SpeC and/or SpeF; EC 4.1.1.17), and a knock-down or knock-out of any native ornithine carbamoyltransferase (ArgI and/or ArgF; EC 2.1.3.3), spermidine synthase (SpeE; EC 2.5.1.16), spermidine acetyltransferase (SpeG; EC 2.3.1.57), glutamate-putrescine ligase (PuuA; EC 6.3.1.11), putrescine:H^+ symporter (PuuP), and RNA polymerase sigma S (sigma 38) factor (RpoS) (Qian *et al.*, 2009).

In one embodiment, the biosynthetic pathway is for producing putrescine and comprises overexpressed or de-regulated N-acetylglutamate synthase (ArgA; EC 2.3.1.1), N-acetylglutamate kinase (ArgB; EC 2.7.2.8), N-acetylglutamylphosphate reductase (ArgC; EC 1.2.1.38), N-acetylornithine aminotransferase/N-succinyldiaminopimelate aminotransferase (ArgD or GabT; EC 2.6.1.11), acetylornithine deacetylase (ArgE; EC 3.5.1.16), ornithine carbamoyltransferase (ArgF or ArgI; EC 2.1.3.3), arginosuccinate synthase (ArgG; EC 6.3.4.5), arginosuccinate lyase (ArgH; EC 4.3.2.1), arginine decarboxylase (SpeA; EC 4.1.1.19), ornithine decarboxylase (SpeC; EC 4.1.1.17), agmatinase (SpeB; EC 3.5.3.11), putrescine:H^+ symporter/putrescine:ornithine antiporter (PotE), and knock-down or knock-out of spermidine synthase (SpeE; EC 2.5.1.16), putrescine:H^+ symporter (PuuP), and glutamate-putrescine ligase (PuuA; EC 6.3.1.11).

In one embodiment, the biosynthetic pathway is for producing cadaverine and comprises an overexpressed lysine decarboxylase (EC 4.1.1.18) and a knock-down or knockout of any native spermidine synthase (SpeE; EC 2.5.1.16), spermidine acetyltransferase (SpeG; EC 2.3.1.57), glutamate-putrescine ligase (PuuA; EC 6.3.1.11), putrescine:H^+ symporter (PuuP), and putrescine/cadaverine aminotransferase (YgjG). In a preferred embodiment, the bacterial cell is an *E. coli* cell and comprises overexpressed lysine decarboxylase (CadA; EC 4.1.1.18) and a knock-down or knock-out of spermidine synthase (SpeE; EC 2.5.1.16), spermidine acetyltransferase (SpeG; EC 2.3.1.57), glutamate-putrescine ligase (PuuA; EC 6.3.1.11), putrescine:H^+ symporter (PuuP), and putrescine/cadaverine aminotransferase (YgjG) (Qian *et al.*, 2011).

In one embodiment, the biosynthetic pathway is for producing HMDA and comprises expression of 3-oxoadipyl-CoA thiolase (PaaJ; EC 2.3.1.174), 3-oxoadipyl-CoA reductase (PaaH), 3-hydroxyadipyl-CoA dehydratase (MaoC), 5-carboxy-2-pentenoyl-CoA reductase (Bcd and EtfAB), adipyl-CoA reductase (aldehyde forming) (Acr1), 6-aminocaproyl-CoA synthase (GabT), 6-aminocaproic acid transaminase (BioW), and hexamethylenediamine transaminase (YgjG) (US 2012/0282661 A1; *e.g.*, Example XVII).

In one embodiment, the biosynthetic pathway is for producing 1,3-diaminopropane and comprises overexpressed aspartate aminotransferase (AspC; EC 2.6.1.1) and phosphoenolpyruvate carboxylase (Ppc; EC 4.1.1.31), a knock-down or knockout of any native 6-phosphofructokinase I (PfkA; EC 2.7.1.-), and expressing mutated versions of aspartate kinase (ThrA; EC 2.7.2.4) and asparate kinase III (LysC; EC 2.7.2.4) that exhibit removal of feedback inhibition.

In one embodiment, the biosynthetic pathway is for producing spermidine and comprises overexpressed or de-regulated N-acetylglutamate synthase (ArgA; EC 2.3.1.1), N-acetylglutamate kinase (ArgB; EC 2.7.2.8), N-acetylglutamylphosphate reductase (ArgC; EC 1.2.1.38), N-acetylornithine aminotransferase/N-succinyldiaminopimelate aminotransferase (ArgD or GabT; EC 2.6.1.11), acetylornithine deacetylase (ArgE; EC 3.5.1.16), ornithine carbamoyltransferase (ArgF or ArgI; EC 2.1.3.3), arginosuccinate synthase (ArgG; EC 6.3.4.5), arginosuccinate lyase (ArgH; EC 4.3.2.1), arginine decarboxylase (SpeA; EC 4.1.1.19), ornithine decarboxylase (SpeC; EC 4.1.1.17), adenosylmethionine decarboxylase (SpeD; EC 4.1.1.50) and spermidine synthase (SpeE; EC 2.5.1.18), and knock-down or knockout of putrescine:H^+ symporter (PuuP), glutamate-putrescine ligase (PuuA; EC 6.3.1.11), and spermidine acetyltransferase (SpeG; EC 2.3.1.57).

In one embodiment, the biosynthetic pathway is for producing ornithine and comprises overexpressed or de-regulated (*e.g.* via knock-down or knockout of ArgR transcriptional dual regulator) N-acetylglutamate synthase (ArgA; EC 2.3.1.1), N-acetylglutamate kinase (ArgB; EC 2.7.2.8), N-acetylglutamylphosphate reductase (ArgC; EC 1.2.1.38), N-acetylornithine aminotransferase/N-succinyldiaminopimelate aminotransferase (ArgD or GabT; EC 2.6.1.11), and acetylornithine deacetylase (ArgE; EC 3.5.1.16), and knock-down or knockout of ornithine carbamoyltransferase (ArgF or ArgI; EC 2.1.3.3) and glutamate 5-kinase (ProB; EC 2.7.2.11) (Hwang *et al.*, 2008)

In one embodiment, the biosynthetic pathway is for producing citrulline and comprises overexpressed or de-regulated (*e.g.* via knock-down or knockout of ArgR transcriptional dual regulator) N-acetylglutamate synthase (ArgA; EC 2.3.1.1), N-acetylglutamate kinase (ArgB; EC 2.7.2.8) or a feedback-resistant mutant thereof, N-acetylglutamylphosphate reductase (ArgC; EC 1.2.1.38), N-acetylornithine aminotransferase/N-succinyldiaminopimelate aminotransferase (ArgD or GabT; EC 2.6.1.11), and acetylornithine deacetylase (ArgE; EC 3.5.1.16), and ornithine carbamoyltransferase (ArgF or ArgI; EC 2.1.3.3), and knock-down or knockout of arginosuccinate synthase (ArgG; EC 6.3.4.5) (Eberhardt *et al.*, 2014).

Additional production pathways that have been employed in microorganisms for the overproduction of putrescine, cadaverine, ornithine, and citrulline are reviewed in Wendisch *et al.* (2016), hereby incorporated by reference in its entirety.

3) Processes

In one aspect, there is provided a process for preparing a recombinant bacterial cell, *e.g.*, an *E. coli* cell. Also provided is a process for improving the tolerance of a bacterial cell, *e.g.*, an *E. coli* cell, to at least one aliphatic polyamine, such as, *e.g.*, putrescine, HMDA, 1,3-diaminopropane, cadaverine, ethylenediamine, spermidine, citrulline, and ornithine. Also provided is a method of identifying a bacterial cell which is tolerant to at least one such aliphatic polyamine. Also provided is a process for preparing a recombinant bacterial cell, *e.g.*, an *E. coli* cell, for producing such an aliphatic polyamine.

These processes may comprise one or more steps of genetically modifying a bacterial cell to knock-down or knock-out one or more endogenous genes of any aspect or embodiment of the Group 1 modifications and/or introducing one or more mutations in the endogenous protein(s) or gene(s) of any Group 2 aspect or embodiment. This can be achieved by, *e.g.*, transforming the bacterial cell with genetic constructs, *e.g.*, vectors, antisense nucleic acids or siRNA, which effect the knock-out or knock-down or which introduce the mutation into the endogenous gene or encode the mutated protein from a transgene.

The genetic constructs, particularly vectors, can also comprise suitable regulatory sequences, typically nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters (*e.g.*, constitutive promoters or inducible promoters), translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures.

Alternatively, bacterial cells can be exposed to selection pressure (as described in the Examples) or to conditions which introduce random mutations in endogenous genes, and bacterial cells which comprise one or more Group 1 and/or Group 2 modifications according to any preceding aspects and embodiments can be identified.

In one specific embodiment, the Group 1 modification is a knock-down or knock-out of one or more endogenous genes selected from *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, ybeX* and *mpl*. In one specific embodiment, the Group 2 modification is a mutation in at least one endogenous protein or gene selected from YgaC, RpsG, MreB, NusA, SspA,

and *argG*, such as YgaC-R43L, RpsG-L157*, MreB-A298V, NusA-L152R, SspA-F83C, MrdB-E254K, RpoD-E575A, RpoC-V401G, RpoB-R637L, MurA-Y393S, RpsA-D310Y, NusA-M204R, MreB-H93N, SpoT-R467H and *argG*-C324A.

The processes may further comprise

- a step of selecting any bacterial cell which has an improved tolerance to at least one aliphatic polyamine, *e.g.*, putrescine, HMDA, 1,3-diaminopropane, cadaverine, ethylenediamine, spermidine, agmatine, citrulline or ornithine at a predetermined concentration, such as at least 25 g/L or higher;

- a step of introducing a recombinant biosynthetic pathway for producing a polyamine, such as, *e.g.*, putrescine, HMDA, 1,3-diaminopropane, cadaverine, ethylenediamine, spermidine, agmatine, citrulline and/or ornithine; or

- both of the above steps, in any order.

Also provided is a method of producing an aliphatic polyamine, comprising culturing the bacterial cell obtained by any one of these methods, or the bacterial cell of any preceding aspect or embodiment, under conditions where the aliphatic polyamine is produced. Typically, these conditions include the presence of a suitable carbon source or mixes of different suitable carbon sources. Non-limiting examples of suitable carbon sources include, *e.g.*, sucrose, D-glucose, D-xylose, L-arabinose, glycerol, as well as hydrolysates produced from cellulosic or lignocellulosic materials. For further details see, *e.g.*, Qian *et al.*, 2009 or 2011.

4) Compositions

A bacterial cell which have an increased tolerance to aliphatic polyamines such as, *e.g.*, putrescine, HMDA, spermidine, agmatine, cadaverine, 1,3-diaminopropane, ethylenediamine, citrulline or ornithine can be useful for the production of such aliphatic polyamines.

In one aspect, there is provided a composition comprising

- an aliphatic polyamine at a concentration of at least 5 g/L, such as at least 15 g/L, such as at least 19 g/L, such as at least 20 g/L, such as at least 25 g/L, such as at least 30 g/L, such as at least 35 g/L, such as at least 38 g/L, such as at least 40 g/L; and

- a plurality of bacterial cells according to any preceding aspect or embodiment.

Preferably, the bacterial cells are of the *Escherichia*, *Bacillus*, *Ralstonia*, *Pseudomonas* or *Corynebacterium* family, such as, *e.g.*, *E. coli* cells, and comprise

a) at least one genetic modification which reduces expression of an endogenous gene selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, ybeX* and *mpl*, or a combination of any thereof;

b) a mutation in at least one of *ygaC, rpsG, mreB, nusA, sspA, mrdB, rpoD, rpoC, rpoB, murA, rpsA, spoT* and *argG* which improves the tolerance of the bacterial cell to putrescine, HMDA, cadaverine,1,3-diaminopropane, spermidine, agmatine, ethylenediamine, citrulline or ornithine; or

c) a combination of a) and b).

5) Bacterial cells

Also provided are strains, clones and other progeny of the bacterial cells of these and other aspects and embodiments. Typically, as used herein, a "strain" typically refers to a group of cells which are descendants of a initial single colony of parent cells whereas a "clone" is a group of cells which are the descendants of an initial genetically modified single parent cell.

Non-limiting examples of bacterial cells suitable for modification according to any one of the aspects and embodiments described herein include bacteria of the *Enterobacteriaceae* or *Corynebacteriaceae* families, particularly the *Escherichia, Bacillus, Ralstonia, Pseudomonas* and *Corynebacterium* genera. In one embodiment, the bacterial cell is an *E. coli* cell, such as a cell of the commercially available and/or fully characterized strains K-12 MG1655, B, BLR, BW25113, BL21, BL21(DE3), K-12 W3110, W, JM109, JM110, REL606, DH1, DH5α, DH10B, C600, S17-1, HB101 or Crooks (ATCC 8739). In another embodiment, the bacterial cell is a *Bacillus* cell, such as a cell of the commercially available and/or fully characterized strains *Bacillus subtilis* 168 and *Bacillus subtilis* PY79. In one embodiment, the bacterial cell is a *Pseudomonas* cell, such as a cell of the commercially available and/or fully characterized strain *Pseudomonas putida* KT2440. In another embodiment, the bacterial cell is a *Ralstonia* cell, such as a cell of the commercially available and/or fully characterized strains *Ralstonia eutropha* H16 and *Ralstonia eutropha* JMP134. In another embodiment, the bacterial cell is a *Corynebacterium* cell, such as a cell of the commercially available and/or fully characterized strains 534 (ATCC 13032), K051, MB001, R, SCgG1, and SCgG2.

While aspect and embodiments relating to bacterial cells herein typically refer to genes or proteins according to their designation in *E. coli*, for bacterial cells of another family or

species, it is within the level of skill in the art to identify the corresponding gene or protein, *i.e.*, the ortholog and/or paralog, in the other family or species, typically by identifying sequences having moderate or high homology to the *E. coli* sequence, optionally taking the function of the protein expressed by the gene and/or the locus of the gene in the genome into account. Table 1 below sets out the function of the protein encoded by each specific gene, the corresponding E.C. number (if applicable), its locus in the *E. coli* K-12 MG1655 genome and the SEQ ID number of the coding sequence.

Table 2 below sets out some examples of homologs in selected organisms, identified in a preliminary and non-limiting analysis. Indeed, homologs of these proteins exist also in other bacteria, and other homologs not identified in this preliminary search can exist in the species listed in Table 2. The skilled person is well-familiar with different searching and/or screening methods for identifying homologs across different species.

Table 1. Protein function and Locus IDs

E. coli designation	Protein function	E.C. number	Locus ID	SEQ ID NO:
proV	ATP-binding subunit of glycine betaine/proline ABC transporter	3.6.3.32	b2677	1
proW	Membrane subunit of glycine betaine/proline ABC transporter	3.6.3.32	b2678	2
proX	Periplasmic binding protein subunit of glycine betaine/proline ABC transporter	3.6.3.32	b2679	3
cspC	Stress protein, member of the CspA family	N/A	b1823	4
ptsP	Phosphoenolpyruvate-protein phosphotransferase PtsP, enzyme I^{Ntr}	2.7.3.9	b2829	5
wbbK	Predicted lipopolysaccharide biosynthesis protein	N/A	b2032	6
yobF	Small protein involved in stress responses	N/A	b1824	7
nagC	NagC DNA-binding transcriptional dual regulator	N/A	b0676	8
nagA	N-acetylglucosamine-6-phosphate deacetylase	3.5.1.25	b0677	9
Rph	RNase PH	2.7.7.56	b3643	10
yicC	Conserved protein	N/A	b3644	42
yjcF	Conserved protein	N/A	b4066	43

iscR	IscR DNA-binding transcriptional dual regulator	N/A	b2531	44
yedP	Predicted phosphatase	N/A	b1955	45
ybeX	Putative transport protein	N/A	b0658	11
MpI	UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-*meso*-diaminoheptanedioate-D-alanine ligase [multifunctional]	6.3.2.45	b4233	12
ygaC	Predicted protein	N/A	b2671	13 (DNA) 14 (protein)
rpsG	30S ribosomal subunit protein S7	N/A	b3341	15 (DNA) 16 (protein)
argG	Argininosuccinate synthase	6.3.4.5	b3172	17 (DNA) 18 (protein)
mreB	Cell wall structural actin-like protein in MreBCD complex; mecillinam resistance protein	N/A	b3251	19 (DNA) 20 (protein)
sspA	Stringent starvation protein A	N/A	b3229	21 (DNA) 22 (protein)
nusA	transcription termination/antitermination L factor	N/A	b3169	23 (DNA) 24 (protein)
mrdB	Rod shape-determining membrane protein; sensitivity to radiation and drugs	N/A	b0634	25 (DNA) 26 (protein)
rpoD	RNA polymerase, sigma 70 (sigma D) factor	N/A	b3067	27 (DNA) 28 (protein)
rpoC	RNA polymerase, β' subunit	2.7.7.6	b3988	29 (DNA) 30 (protein)
rpoB	RNA polymerase, β subunit	2.7.7.6	b3987	31 (DNA) 32 (protein)
murA	UDP-*N*-acetylglucosamine enolpyruvyl transferase	2.5.1.7	b3189	33 (DNA) 34 (protein)
rpsA	30S ribosomal subunit protein S1	N/A	b0911	35 (DNA) 36 (protein)
spoT	Guanosine 3'-diphosphate 5'-triphosphate 3'-diphosphatase [multifunctional]	3.1.7.2	b3650	37 (DNA) 38 (protein)
pyrE	Orotate phosphoribosyltransferase	2.4.2.10	b3642	39 (DNA) 40 (protein)

pyrE/rph intergenic region	-	-	-	41

Table 2A and 2B. Homologs or orthologs identified by protein BLAST (BLASTP) of E. coli K-12 MG1655 proteins against protein databases from selected reference organisms. Hits with the largest e-value are shown, and hits are only shown when the e-value < 1.0. Hit proteins with e-value < 0.1 (non-italicized) are deemed the most probable of having the same or similar function as the E. coli protein.

Table 2A:

Protein (# of residues)	***B. subtilis* 168**	***P. putida* KT2440**
ProV (400 aa)	51% identity (390 aa) "glycine/betaine ABC transporter ATP-binding protein" (NP_388180.2); 37% identity (360 aa) "glycine betaine/carnitine/choline ATP-binding protein OpuCA" (NP_391263.1); 36% identity (352 aa) "choline transport ATP-binding protein OpuBA" (NP_391253.1)	51% identity (291 aa) "glycine betaine/L-proline ABC transporter ATP-binding subunit" (NP_742461.1); 51% identity (222 aa) "glycine betaine/L-proline ABC transporter ATPase/permease fusion protein" (NP_744918.1); 36% identity (352 aa) "glycine betaine/L-proline ABC transporter ATPase" (NP_743029.1)
ProW (354 aa)	48% identity (275 aa) "glycine betaine transport system permease protein OpuAB" (NP_388181.1); 31% identity (186 aa) "choline transport system permease protein OpuBB" (NP_391252.1); 30% identity (187 aa) "glycine betaine/carnitine/choline transport system permease protein OpuCB" (NP_391262.1); 30% identity (187 aa) "glycine betaine/carnitine/choline transport system permease protein OpuCD" (NP_391260.1)	40% identity (265 aa) "glycine betaine/L-proline ABC transporter permease" (NP_742462.1); 53% identity (206 aa) "binding-protein-dependent transport system inner membrane protein" (NP_745696.1); 42% identity (259 aa) "glycine betaine/L-proline ABC transporter ATPase/permease fusion protein" (NP_744918.1)
ProX (330 aa)	25% identity (124 aa) "glycine betaine-binding protein OpuAC" (NP_388182.1)	27% identity (155 aa) "glycine betaine ABC transporter substrate-binding protein" (NP_745695.1); 24% identity (200 aa) "glycine/betaine ABC transporter substrate-binding protein (NP_744919.1); 21% identity (327 aa) "glycine betaine-binding protein" (NP_742246.1)

CspC (70 aa)	67% identity (64 aa) "cold shock protein CspB" (NP_388791.1); 59% identity (64 aa) "cold shock protein CspD" (NP_390076.1); 60% identity (62 aa) "cold shock protein CspC" (NP_388393.1)	58% identity (64 aa) "cold shock protein CspA" (NP_744611.1); 60% identity (67 aa) "cold-shock domain-contain protein" (NP_743146.1); 63% identity (61 aa) "cold-shock domain-contain protein" (NP_743260.1); 50% identity (68 aa) "cold shock protein CspA" (NP_743679.1); 55% identity (64 aa) "cold-shock domain-contain protein, partial" (NP_743369.1); 54% identity (61 aa) "cold-shock protein CspD" (NP_746140.1)
PtsP (749 aa)	33% identity (572 aa) "phosphoenolpyruvate-protein phosphotransferase" (NP_389274.2)	43% identity (729 aa) "protein PtsP" (NP_747246.1); 38% identity (575 aa) "phosphoenolpyruvate-protein phosphotransferase" (NP_742954.1)
WbbK (373 aa)	26% identity (110 aa) "glycosyltransferase YpjH" (NP_390127.1)	28% identity (111 aa) "glycosyl transferase WbpY" (NP_743956.1)
YobF (48 aa)	-	42% identity (31 aa) "preprotein translocase subunit SecD" (NP_742996.1)
NagC (407 aa)	25% identity (358 aa) "transcriptional regulator" (NP_389641.2)	-
NagA (383 aa)	32% identity (380 aa) "N-acetylglucosamine-6-phosphate deacetylase" (NP_391381.1)	22% identity (391 aa) "guanine deaminase" (NP_746397.1)
Rph (228 aa)	58% identity (222 aa) "ribonuclease PH" (NP_390715.1)	69% identity (228 aa) "ribonuclease PH" (NP_747395.1)
PyrE (213 aa)	25-34% identity (in stretches) "orotate phosphoribosyl-transferase" (NP_389439.1)	67% identity (213 aa) "orotate phosphoribosyl-transferase" (NP_747392.1)
YbeX (293 aa)	33% identity (253 aa) "hypothetical protein BSU31300" (NP_391008.2); 32% identity (260 aa) "hypothetical protein BSU24750" (NP_390355.1); 33% identity (252 aa) "hypothetical protein BSU26610" (NP_390538.1); 33% identity (257 aa) "hypothetical protein BSU09590" (NP_388840.1); 29% identity (279 aa) "hypothetical protein BSU9550" (NP_388836.1)	53% identity (268 aa) "hypothetical protein PP_4789" (NP_746894.1)
Mpl (458 aa)	30% identity (386 aa) "UDP-N-acetylmuramate—L-alanine ligase" (NP_390857.1)	59% identity (449 aa) "UDP-N-acetylmuramate" (NP_742710.1); 29% identity (469 aa) "UDP-N-acetylmuramate—L-alanine ligase (NP_743497.1)
YgaC (115 aa)	*29% identity (55 aa) "transglycosylase YomI" (NP_390018.2)*	*29% identity (51 aa) "penicillin amidase" (NP_745045.1)*
RpsG (180 aa)	56% identity (156 aa) "30S ribosomal protein S7" (NP_387992.2)	71% identity (156 aa) "30S ribosomal protein S7" (NP_742616.1)
ArgG (448 aa)	29% identity (414 aa) "arginosuccinate synthase" (NP_390823.1)	26% identity (409 aa) "arginosuccinate synthase" (NP_743249.1)

MreB (348 aa)	58% identity (337 aa) "rod shape-determining protein MreB" (NP_390681.2)	80% identity (347 aa) "rod shape-determining protein MreB" (NP_743094.2)
SspA (212 aa)	-	57% identity (200 aa) "stringent starvation protein A" (NP_743480.1)
NusA (496 aa)	40% identity (343 aa) "transcription termination/antitermination protein NusA" (NP_389542.1)	64% identity (493 aa) "transcription elongation factor NusA" (NP_746821.1)
MrdB (371 aa)	32% identity (344 aa) "stage V sporulation protein E" (NP_389404.1); 30% identity (352 aa) "lipid II flippase FtsW" (NP_389368.1); 30% identity (289 aa) "rod shape-determining protein RodA" (NP_391691.1)	53% identity (364 aa) "rod shape-determining protein RodA" (NP_746911.1)
RpoD (614 aa)	69% identity (238 aa) "RNA polymerase sigma factor RpoD" (NP_390399.2)	67% identity (610 aa) "RNA polymerase sigma factor RpoD" (NP_742554.1)
RpoC (1407 aa)	50% identity (1134 aa) "DNA-directed RNA polymerase subunit beta'"	75% identity (1399 aa) "DNA-directed RNA polymerase subunit beta'" (NP_742614.1)
RpoB (1342 aa)	59% identity (533 aa) "DNA-directed RNA polymerase subunit beta"	72% identity (1360 aa) "DNA-directed RNA polymerase subunit beta" (NP_742613.1)
MurA (420 aa)	50% identity (422 aa) "UDP-N-acetylglucosamine-1 carboxyvinyltransferase 1" (NP_391557.1); 45% identity (418 aa) "UDP-N-acetylglucosamine-1 carboxyvinyltransferase 2" (NP_391591.2)	61% identity (421 aa) "UDP-N-acetylglucosamine 1-carboxyvinyltransferase" (NP_743125.1)
RpsA (558 aa)	39% identity (338 aa) "30S ribosomal protein S1 homolog" (NP_390169.1)	74% identity (554 aa) "30S ribosomal protein S1" (NP_743928.2)
SpoT (702 aa)	40% identity (719 aa) "GTP pyrophosphokinase" (NP_390638.2)	55% identity (701 aa) "(p)ppGpp synthetase I SpoT/RelA" (NP_747403.1); 37% identity (681 aa) "(p)ppGpp synthetase I SpoT/RelA" (NP_743813.1)

Table 2B:

Protein (# of residues)	***Ralstonia eutropha* H16**	***Corynebacterium glutamicum* ATCC 13032**
ProV (400 aa)	40% identity (214 aa) "ABC transporter ATPase" (YP_724876.1); 37% identity (239 aa) "ABC transporter ATPase" (YP_726702.1); 33% identity (354 aa) "ABC transporter ATPase" (YP_725457.1); 39% identity (263 aa) "ABC transporter ATPase" (YP_725326.1); 39% identity (198 aa) "ABC	34-40% identity (194-283 aa) "ABC transporter ATPase" or "ABC transporter duplicated ATPase" (NP_599870.1, NP_601662.1, NP_599673.1, NP_599959.1, NP_601157.1, NP_600605.1, NP_600550.1, NP_601199.1, NP_602190.1, NP_599469.1, NP_601634.1, NP_601634.1,

	transporter ATPase" (YP_726845.1); 39% identity (223 aa) "ABC transporter ATPase" (YP_724565.1); 38% identity (232 aa) "ABC transporter ATPase" (YP_727745.1)	NP_601523.1, NP_601854.1, NP_600446.1, NP_600085.1, NP_600031.1, NP_600677.1)
ProW (354 aa)	34% identity (194 aa) "ABC transporter permease" (YP_725456.1); 32% identity (177 aa) "ABC-type transporter, fused periplasmic and permease components" (YP_726088.1); 31% identity (152 aa) "ABC transporter permease" (YP_725454.1); 29% identity (175 aa) "ABC transporter permease" (YP_726844.1)	25-32% identity (124-232 aa) "ABC transporter permease" (NP_600676.1, NP_600445.1, NP_601771.1, NP_599955.1)
ProX (330 aa)	*29% identity (75 aa) "RND superfamily exporter" (YP_725351.1)*	-
CspC (70 aa)	61% identity (61 aa) "cold shock protein, DNA-binding" (YP_727497.1)	66% identity (65 aa) "cold shock protein" (NP_599560.1); 62% identity (65 aa) "cold shock protein" (NP_599426.1); 34% identity (64 aa) "cold shock protein" (NP_600049.1)
PtsP (749 aa)	35% identity (497 aa) "phosphoenolpyruvate-protein kinase (PTS system EI component)" (YP_724845.1); 32% identity (567 aa) "protein-N(pi)-phosphohistidine-sugar phosphotransferase II ABC" (YP_724830.1)	31% identity (539 aa) "phosphoenolpyruvate-protein kinase" (NP_601139.1)
WbbK (373 aa)	31% identity (103 aa) "glycosyltransferase group 1" (YP_726331.1); 25% identity (142 aa) "glycosyltransferase" (YP_727345.1)	29% identity (75 aa) "group 1 glycosyltransferase" (NP_599714.1); 24% identity (139 aa) "glycosyltransferase" (NP_601390.1)
YobF (48 aa)	-	-
NagC (407 aa)	35% identity (48 aa) "ArsR family transcriptional regulator" (YP_726640.1)	29% identity (273 aa) "glucose kinase" (NP_601389.1); 22% identity (186 aa) "transcriptional regulator" (NP_601847.1); 21% identity (327 aa) "transcriptional regulator" (NP_599261.2)
NagA (383 aa)	33% identity (330 aa) "N-acetylglucosamine-6-phosphate deacetylase" (YP_724833.1)	24% identity (345 aa) "N-acetylglucosamine-6-phosphate deacetylase" (NP_601845.2)
Rph (228 aa)	62% identity (221 aa) "ribonuclease PH" (YP_725462.1)	59% identity (217 aa) "ribonuclease PH" (NP_601703.2)
PyrE (213 aa)	56% identity (215 aa) "orotate phosphoribosyl-transferase" (YP_724744.1)	29% identity (139 aa) "orotate phosphoribosyl-transferase" (NP_601967.1)
YbeX (293 aa)	45% identity (259 aa) "Mg2+/Co2+ transporter" (YP_725043.1); 26% identity (269 aa) "Mg2+/Co2+ transporter" (YP_725283.1)	34% identity (259 aa) "hypothetical protein NCgl2206" (NP_601486.1); 32% identity (225 aa) "hypothetical protein NCgl1393" (NP_600666.1); 27% identity (307 aa) "hypothetical protein NCgl1147" (NP_600420.1)

Mpl (458 aa)	60% identity (463 aa) "UDP-N-acetylmuramate-L-alanine ligase" (YP_727610.1); 28% identity (476 aa) "UDP-N-acetylmuramate—L-alanine ligase" (YP_727714.1)	28% identity (484 aa) "UDP-N-acetylmuramate—L-alanine ligase" (NP_601359.1)
YgaC (115 aa)	*32% identity (37 aa) "glutathione S-transferase" (YP_727111.1)*	*32% identity (99 aa) "N-acetyl-gamma-glutamyl-phosphate reductase" (NP_600613.1)*
RpsG (180 aa)	65% identity (156 aa) "30S ribosomal protein S7" (YP_727929.1)	57% identity (148 aa) "30S ribosomal protein S7" (NP_599739.1)
ArgG (448 aa)	33% identity (61 aa) "PP-loop superfamily ATPase" (YP_727271.1)	29% identity (398 aa) "arginosuccinate synthase" (NP_600619.1)
MreB (348 aa)	72% identity (350 aa) "rod shape-determining protein MreB" (YP_724633.1)	27% identity (220 aa) "molecular chaperone DnaK" (NP_601992.1)
SspA (212 aa)	46% identity (203 aa) "stringent starvation protein A" (YP_727831.1)	*56% identity (16 aa) "hypothetical protein NCgl2333" (NP_601617.1)*
NusA (496 aa)	50% identity (494 aa) "transcription elongation factor NusA" (YP_726771.1)	32% identity (352 aa) "transcriptional elongation factor NusA" (NP_601193.1)
MrdB (371 aa)	49% identity (364 aa) "rod-shape-determining protein RodA" (YP_724637.1)	30% identity (359 aa) "cell division membrane protein" (NP_601361.1); 28% identity (295 aa) "cell division membrane protein" (NP_599296.1)
RpoD (614 aa)	56% identity (618 aa) "RNA polymerase sigma factor RpoD" (YP_727172.1); 47% identity (632 aa) "DNA-directed RNA polymerase subunit (RpoD)" (YP_726126.1)	60% identity (241 aa) "RNA polymerase sigma factor" (NP_601117.2)
RpoC (1407 aa)	67% identity (1397 aa) "DNA-directed RNA polymerase subunit beta'" (YP_727932.1)	50% identity (819 aa) "DNA-directed RNA polymerase subunit beta'" (NP_599734.1)
RpoB (1342 aa)	66% identity (1370 aa) "DNA-directed RNA polymerase subunit beta" (YP_727933.1)	56% identity (616 aa) "DNA-directed RNA polymerase subunit beta" (NP_599733.1)
MurA (420 aa)	60% identity (417 aa) "UDP-N-acetylglucosamine 1-carboxyvinyltransferase" (YP_727854.1)	46% identity (417 aa) "UDP-N-acetylglucosamine 1-carboxyvinyltransferase" (NP_601757.1); 31% identity (425 aa) "UDP-N-acetylglucosamine enoylpyruvyl transferase" (NP_599603.1)
RpsA (558 aa)	67% identity (529 aa) "30S ribosomal protein S1" (YP_725313.1)	46% identity (340 aa) "30S ribosomal protein S1" (NP_600575.1)
SpoT (702 aa)	47% identity (720 aa) "GTP pyrophosphokinase" (YP_725468.1); 36% identity (674 aa) "GTP pyrophosphokinase" (YP_725845.1)	38% identity (723 aa) "guanosine polyphosphate pyrophospho-hydrolase/ synthetase" (NP_600866.1); 33% identity (129 aa) "guanosine polyphosphate pyrophosphohydrolase/ synthetase" (NP_600534.2)

So, in one aspect, there is provided a bacterial cell according to any one of the preceding aspects and embodiments, wherein each recited gene is instead (i) a gene encoding the corresponding protein in the Table above, (ii) a gene located at the corresponding locus, or (iii) both.

In particular, without being limited to theory, improved tolerance toward polyamines can be achieved by genetic modifications which

- reduce the transporter-mediated import of polyamines, *e.g.*, via the glycine betaine/proline ABC transporter or YbeX predicted transporter;
- reduce cellular stresses imposed by a high level of extracellular binding of polyamines to the cell surface, via modifications to outer membrane saccharides (*e.g.* via the lipopolysaccharide biosynthesis protein WbbK) or modifications to cell shape that increase the surface area to volume ratio (*e.g.* via mutations in MreB, MurA, or MrdB)
- modulate the NagC regulon either directly through a reduction in levels of NagC, or indirectly through reduced levels of NagA (which deacetylates N-acetyl-D-glucosamine 6-phosphate, a molecule which binds to NagC and causes dissociation from DNA);
- restore improved expression of orotate phosphoribosyltransferase (PyrE), *e.g.* by deletion of *rph*;
- improve general stress resistance toward stresses imposed by polyamines, *e.g.*, by reduced levels of CspC and/or YobF; and/or
- alter processes of cell wall recycling, *e.g.* by reduced levels of Mpl, NagC, or NagA.
- reduce the effect of polyamines on the nucleoid by restoring levels of nucleoid-associated proteins such as H-NS and/or StpA, via mutations in SspA or YgaC.
- reduce the effect of polyamines on altered transcription termination by mutations that reduce the activity of the NusA.
- reduce the effects of excessive polyamine bound to ribosomes via mutations in RpsG and RpsA
- reduce the efflux of Mg^{2+} cations or other divalent cations, which compete for nucleic acid binding with polyamines, *e.g.* via elimination of the Mg^{2+}/divalent cation efflux transporter YbeX
- reduce the intracellular levels of polyamines, *e.g.* by alteration of transport protein levels due to alterations in cell shape, alterations in the cell wall, and reduced levels of cation efflux transporters that would otherwise balance cellular charge with imported polyamines, *e.g.* via mutations in MreB, MurA, MrdB, Mpl, NagC, NagA, or YbeX.

So, in one embodiment, the bacterial cell has a genetic modification which reduces the expression of one or more endogenous proteins selected from the group consisting of

- an ATP-binding subunit of glycine betaine/proline ABC transporter
- a membrane subunit of glycine betaine/proline ABC transporter
- a periplasmic binding protein subunit of glycine betaine/proline ABC transporter
- a stress-protein member of the CspA family
- a phosphoenolpyruvate-protein phosphotransferase PtsP, enzyme INtr
- a lipopolysaccharide biosynthesis protein
- a small protein involved in stress responses
- a NagC DNA-binding transcriptional dual regulator
- a N-acetylglucosamine-6-phosphate deacetylase
- an RNase PH
- a UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-*meso*-diaminoheptanedioate-D-alanine ligase
- a transport protein involved in Mg^{2+}/Co^{2+} or other divalent cation efflux

In addition, without being limited to theory, improved tolerance toward polyamine can also be achieved by genetic modifications which

- reduce ribosomal frameshifting during translation of proteins under stress conditions imposed by polyamines, *e.g.*, via a genetic modification in *rpsG*;
- reduce levels of one or more precursors for intracellular biosynthesis of polyamines, *e.g.*, via a genetic modification in *argG* or *ygaC*;
- alter the cytoskeletal scaffold to increase the surface to volume ratio of the cell, *e.g.* via a genetic modification in *mreB*, *mrdB*, or *murA*;
- reducing the effect of polyamines on altered transcription termination induced by high concentrations of polyamines, *e.g.*, via a genetic modification in *nusA*; and/or
- reduce the effect of polyamines on the nucleoid by restoring levels of nucleoid-associated proteins such as H-NS and/or StpA, via genetic modifications in *sspA* or *ygaC*.
- reduce cellular stresses imposed by a high level of extracellular binding of polyamines to the cell surface, via modifications to cell shape that increase the surface area to volume ratio, *e.g.* via genetic modifications in *mreB, mrdA* or *murA*.
- reduce intracellular levels of polyamines via reduced alteration of transport protein levels due to alterations in cell shape or alterations in the cell wall, *e.g.* via mutations in *mreB, murA, mrdB, or mpl*.

- reduce the effects of excessive polyamine bound to ribosomes, *e.g.* via genetic modifications in *rpsG* and *rpsA*

In one specific embodiment, the bacterial cell further comprises a recombinant biosynthetic pathway for producing a polyamine, such as, *e.g.*, putrescine, HMDA, spermidine, agmatine, 1,3-diaminopropane, cadaverine, ethylenediamine, citrulline and/or ornithine. In one additional embodiment, the bacterial cell is of the *Corynebacterium* genera. In one additional embodiment, the bacterial cell is of the *Escherichia* genera. In one additional embodiment, the bacterial cell is of the *Bacillus* genera. In one additional embodiment, the bacterial cell is of the *Ralstonia* genera. In one additional embodiment, the bacterial cell is of the *Pseudomonas* genera.

EXAMPLE 1

Methods

1) Screening for tolerance in wild-type cells

Escherichia coli K-12 MG1655 was grown overnight in M9 minimal medium + 1% glucose and subcultured the following morning to an initial OD600 of 0.05 in M9 + 1% glucose. Cells were grown to mid-exponential phase (OD600 0.7-1.0) and were back-diluted with fresh medium to an OD600 of 0.7. The diluted cells were used to inoculate M9 + 1% glucose containing varying concentrations of putrescine dihydrochloride or HMDA dihydrochloride, and growth was measured in FlowerPlates in a Biolector microbioreactor system (m2p-labs) at 37°C with 1000 rpm shaking. The culture volume in each well was 1.4 mL.

2) Adaptive laboratory evolution of tolerant strains

Based on the screening results, *E. coli* K-12 MG1655 was grown overnight in M9 minimal medium and 150 µL was transferred the next day into 8 tubes containing 15 mL of M9 + 1% glucose + 25 g/L putrescine on a Tecan Evo robotic platform custom-designed for performing adaptive laboratory evolutions (ALE). Cells were cultured on a 37°C heat block with stirring by magnetic stir bars. Culture OD600 was monitored at times determined by a predictive custom script, and when the OD600 reached approximately 0.3, 150 µL of culture was inoculated into a new tube with the same media concentration. Instrument downtime would occasionally result in cells overgrowing to saturation or an OD600 greater than 0.3, and reinoculations were occasionally performed from cryogenic stocks of the population. When the growth rate was observed to substantially increase, the media concentration was changed. These concentration changes for putrescine were to 30 g/L and 38 g/L, while the

changes for HMDA were to 30 g/L, 35 g/L, and 38 g/L. Approximately 100 µL of each population (8 per chemical) were plated on LB agar and incubated at 37°C overnight.

3) Primary screening of ALE isolates

Five colonies from wild-type K-12 MG1655 and 10 individual colonies deriving from each population were inoculated into 300 µL M9 + 1% glucose in 96 well deepwell plates and incubated in a 300 rpm plate shaker at 37°C. The next day, cells were diluted 10X in M9 + 1% glucose and 30 µL was transferred into clear-bottomed 96 well half-deepwell plates (with rectangular wells) containing M9 + 1% glucose and M9 + 1% glucose + 42.22 g/L putrescine or HMDA, such that the final concentration of putrescine or HMDA was 38 g/L. In addition, cryogenic glycerol stocks of the overnight culture were saved in a 96 well plate format. Half deepwell plates were incubated at 37°C with 225 rpm shaking in a Growth Profiler (Enzyscreen), with optical scans of the plates taken at 15 minute intervals. Green pixel values integrated over a 1 mm diameter circular area in each well were converted to OD600 values using a previously determined calibration between OD600 and green pixel values. Resulting growth curves were visually inspected for isolates exhibiting the most robust or unique growth patterns within each population. In general, it was attempted to select three isolates per population for further analysis, however isolates from some populations exhibited poor growth and were not considered further.

4) Secondary screening of ALE isolates

Selected isolates from the primary screen were restruck onto LB agar from the cryogenic stock made from the overnight culture plate for the primary screen. Five K-12 MG1655 colonies and three individual colonies from each isolate were inoculated as biological replicates into a new 96 well deepwell plate containing 300 µL of M9 + 1% glucose, and grown overnight as for the primary screen. The next day, a cryogenic stock and half deepwell plates containing M9 + 1% glucose with or without putrescine or HMDA were inoculated using the plate of overnight cultures, and growth was measured as described for the primary screen. Resulting growth curves were visually inspected for isolates exhibiting robust and reproducible growth between replicates in high concentrations of putrescine or HMDA.

5) Re-sequencing of ALE isolates

A total of 20 isolates were selected from the secondary screen for whole-genome resequencing. An individual colony was taken from the LB agar plates prepared following the primary screen, inoculated into 2 mL LB, and grown overnight at 37°C in a 250 rpm shaker.

The following morning, 0.5 mL of cells were transferred to microcentrifuge tubes and centrifuged at 16000 x g for 2 minutes. The supernatant was removed and pellets were stored at -20°C until further processing. Genomic DNA was extracted from thawed cell pellets using a PureLink genomic DNA extraction kit, with further concentration and purification performed by ethanol precipitation. To generate libraries for sequencing, the Illumina TruSeq Nano kit was used according to the manufacturers' directions using an input quantity of 200 ng of genomic DNA from each isolate. Sequencing was performed on an Illumina MiSeq sequencer, with a minimum 20X average genomic coverage ensured for each isolate based on the number of reads. Fastq output files were analyzed for variants compared to the K-12 MG1655 reference genome (accession number NC_000913.3) using breseq.

6) Construction of gene knockouts

Probable important losses-of-function (Group 1) were determined by identifying genes across all isolates that harbored mutations, especially those occurring in multiple populations, and by the presence of at least one mutation that either generated a premature stop codon, a frameshift mutation, or the presence of an insertion element sequence within the gene. For those genes, the corresponding knockout strain from the Keio collection of single knockout mutants (where each gene is replaced with a cassette consisting of a kanamycin resistance gene flanked by FRT sites) was used as a donor strain for P1vir phage transduction. Briefly, the Keio strain was grown to early exponential phase in LB + 5 mM CaCl2 and 80 µL of a P1vir stock raised on K-12 MG1655 was added. After significant lysis was observed after 1.5 to 2 hours, the lysate was filter-sterilized to remove cells and stored at 4°C. Strain K-12 MG1655 was grown overnight in LB + 5 mM CaCl2 and 100 µL of the overnight culture was mixed with 100 µL of the P1vir lysate of the Keio collection mutant, and the mixture was incubated at 37°C without shaking for 20 minutes. The entire mixture was then plated on LB agar containing 1.25 mM sodium pyrophosphate as a chelating agent and 25 µg/mL kanamycin. One colony was then restruck on LB + 1.25 mM Na2P4O7 + 25 µg/mL kanamycin plate and analyzed for presence of the Keio cassette in place of the wild-type gene by colony PCR. When further knockouts were constructed in the same strain, the Keio cassette was flipped out to generate a scar sequence such that KanR marker could be recycled. This was performed by transforming with pCP20, which constitutively expresses a flippase recombinase, and plating cells on LB agar + 100 µg/mL ampicillin and incubating at 30°C. The next day, one or more colonies were tested by colony PCR for loss of the Keio cassette, and successful mutants were then cured of pCP20 by elevated temperature curing at 40°C. Strains were verified to be cured of plasmid by plating on LB agar + 100 µg/mL ampicillin and incubation at 30°C. P1vir transductions were then performed using these mutant strains as recipients.

7) Biolector growth screening of evolved isolates and reconstructed mutants

Biological triplicate cultures of each strain were grown to saturation overnight in 96 well deepwell plates containing 300 µL M9 + 1% glucose. The next day, cells were diluted 1:10 in deionized water in a clear 96 well plate and the OD600 was measured on a BioTek plate reader. 48 well FlowerPlates containing a final volume of 1.4 mL of M9 + 1% glucose (plus relevant chemical) were inoculated to OD600 0.03 (with plate reader pathlength, 200 µL volume) with the overnight culture and sealed with Breathseal film. Light backscatter intensity was monitored in a Biolector microbioreactor system at 37°C with 1000 rpm shaking.

8) Keio collection screening for Group 1 (loss-of-function) mutations

For primary screening, Keio collection mutants were inoculated directly from a cryogenic stock of the Keio collection into 300 µL LB medium containing 25 µg/mL kanamycin in 96 well deepwell plates and grown at 37°C with 300 rpm shaking overnight. The Keio background strain, BW25113, was also inoculated into wells of this plate as a control. A cryogenic stock was made from each plate, and the cryogenic stock was replica plated into another 96 well deepwell plate containing 300 µL M9 + 1% glucose and grown overnight. The next day, cells were inoculated 1:100 into clear bottomed 96 well half-deepwell plates containing M9 + 1% glucose plus 32 g/L and 38 g/L putrescine or HMDA and cultivated in a Growth Profiler as previously described for screening of ALE isolates.

As a secondary screen, promising Keio collection mutants were struck on LB + 25 µg/mL kanamycin from the cryogenic stock plate prepared during primary screening above and biological triplicate colonies were inoculated into a 96 well deepwell plate containing 300 µL M9 + 1% glucose. The next day, cells were inoculated into plates for cultivation on the Growth Profiler as described above.

9) Conjugation-mediated genome shuffling

To assist in the identification of causative mutations in selected evolved isolates, a technique was employed by conjugating the wild-type background strain K-12 MG1655 with Hfr ("High frequency of recombination") mutants of the evolved isolates. In order to generate the Hfr mutants of evolved isolates, conjugations were first performed between evolved isolates transformed with pBAD30 (confers ampicillin resistance) with strains CAG60452 and CAG60453 (obtained from Prof. Jeffrey Barrick, University of Texas at Austin) which are 2,6-diaminopimelic acid auxotrophs that harbor integrated F plasmids containing a spectinomycin resistance marker at genomic loci at opposing ends of the genome. Following conjugation,

evolved isolates harboring the integrated F plasmid were obtained by plating on LB agar containing both spectinomycin and ampicillin. These strains were subsequently conjugated over 1-2 days with 140 rpm shaking at 37°C with K-12 MG1655 harboring pACYCDuet-1 (confers chloramphenicol resistance). The resulting conjugation mixture was plated on M9 agar plates containing 25 µg/mL chloramphenicol plus 38 g/L putrescine or 38 g/L HMDA, depending on the evolved isolate employed, to isolate only the wild-type strain. Larger colonies appearing either independently or overlaid on a background of slower growing, likely wild-type cells were picked and restruck on new plates containing chloramphenicol and putrescine or HMDA. Individual isolates were tested for their growth phenotype in biological triplicates and selected isolates were whole-genome resequenced.

10) Multiplex automated genome engineering (MAGE)

Genomic point mutants were generated using MAGE (REF), which involves multiple cycles of electroporation of cells expressing the β protein of λ Red recombinase with single stranded DNA oligonucleotides. The single-stranded oligonucleotides are believed to behave like Okazaki fragments during DNA replication, and their use enables a high enough efficiency of allelic replacement to preclude needing to select for cells that received the mutation.

In this work, K-12 MG1655 was transformed with pMA7SacB (manuscript in revision), a plasmid that harbors the β subunit of λ Red recombinase and Dam (which we have shown in the manuscript in revision to enable low off-target mutation rates and preclude the use of mutator strains as is usually done when performing MAGE) under control of an arabinose-inducible promoter, and SacB to enable removing the plasmid by sucrose counterselection following the identification of a desired mutant. K-12 MG1655/pMA7SacB was grown in 15 mL of LB medium plus 100 µg/mL ampicillin to mid-exponential phase at 37°C, induced for 10 minutes with 0.2% L-arabinose, chilled in an ice water bath, and washed and concentrated 3 times with autoclaved chilled MilliQ water in a typical electrocompetent cell preparation. 50 pmol of oligonucleotide was added to a 50 µL aliquot of cells in a 1 mm gap electroporation cuvette, and cells were electroporated at 1.8 kV. Cells were immediately recovered in 1 mL LB and the entire volume of cells was used to inoculate the next 15 mL LB culture. Cells were grown to mid-exponential phase and the remainder of the procedure repeated, and recovered cells following electroporation were outgrown overnight to allow full genome segregation. The following morning, cells were plated on LB medium.

Colonies appearing on LB medium were then screened for the presence of the desired introduced mutation. Colonies were resuspended in water for use as a template in a quantitative PCR (qPCR) with a HotStart Taq master mix containing SYBR Green. To achieve discrimination of a mutated base via the cycle threshold, both wild-type and mutant forward

primers were designed and run as separate reactions with the same reverse primer binding approximately 80-100 bp downstream of the mutation. The mutant forward primer had the last base designed to be complementary to the mutated base and an additional mutation at the -3 position from the 3' end of the primer such that primer binding would be maximally destabilized with the wild-type base. The wild-type primer typically had the -3 position from the 3' end of the primer mutated to offer additional destabilization with the mutant base. This allowed discrimination of the desired mutant or wild-type base for each screened isolate by qualitatively observing a reversal in the fluorescence vs. cycle threshold curves by qPCR with the two primer sets. Individual isolates were verified to have the desired mutant sequence in the genome with no adjacent off-target mutations by Sanger sequencing.

11) Cross-compound tolerance screening

96 well deepwell plates containing 300 µL of M9 + 1% glucose were inoculated directly from cryogenic stocks made from precultures for the secondary screening of ALE isolates and were grown overnight at 37°C with 300 rpm shaking. The next day, cells were diluted 1:100 into 96 well half-deepwell plates containing the following final concentrations of each chemical in M9 + 1% glucose:

Butanol	1.4% v/v
Glutarate	40 g/L
p-coumarate	7.5 g/L
Putrescine	32 g/L
HMDA	32 g/L
Adipate	45 g/L
Isobutyrate	7.5 g/L
Hexanoate	3 g/L
Octanoate	8 g/L
2,3-butanediol	6% v/v
1,2-propanediol	6% v/v
sodium chloride	0.6 M

Plates were cultivated in a Growth Profiler for 48 hours as described for screening of ALE isolates. Green pixel integrated values from each well were converted to OD600 values using a calibration curve and the resulting OD600 vs. elapsed time data was processed using custom scripts to determine the time required for each culture to reach an OD of 1.0 (tOD1). This value is a combined measure of growth rate and lag time in each culture. The median value was taken for biological triplicates of each isolate and was normalized to the median tOD1 for K-12 MG1655 controls (5 replicates). The ratio of tOD1(evolved)/tOD1(wild-type) is presented.

12) Flow cytometry

In preliminary tests, overnight precultures of each strain picked from single colonies on LB plates were grown in M9 + 1% glucose to saturation overnight. Cells were diluted 1:100 directly into 100 µL phosphate buffered saline (PBS) and 1 µL of 10X diluted SYTOX Green was added. Flow cytometry was performed on a Fortessa flow cytometer (Becton Dickenson) with forward and side scatter channels set to 220 V. Events were thresholded with a minimum forward scatter value of 200.

In later screens of all strains, overnight cultures were replica plated from stored cryogenic plates containing all secondary screened PUTR isolates into 300 µL M9 + 1% glucose in 96 well deepwell plates. Cells were grown to saturation overnight and subcultured into new cultures containing M9 or M9 + 38 g/L putrescine (both plus 1% glucose). At different timepoints that represented exponential or stationary phase for the majority of strains in each condition, 100 µL of each culture was harvested, spun down, the media was removed, and resuspended in 100 µL of PBS. Resuspended cells were diluted 1:100 in PBS with SYTOX Green added as above, and cells were analyzed as described above.

13) Phase contrast microscopy

Cells were grown to exponential phase as described for flow cytometry. Glass slides were prepared with a thin layer of LB agar and a small volume of cell culture was spotted onto the agar and covered with a glass cover slip. Images were obtained under phase contrast on a Leica Microsystems fluorescence microscope with white light backlighting and 1000X total magnification with a 100X oil immersion lens.

14) Construction of production strains

Strain XQ52 and plasmid p15SpeC were generously provided by S. Y. Lee (Qian *et al.*, 2009). For screening of putrescine production in ALE evolved isolates, p15SpeC was transformed into each isolate and K-12 MG1655 as a control. Briefly, cells were grown to exponential phase in LB medium, transferred to an ice water bath, centrifuged at 4000 x g for 5 minutes in a refrigerated microcentrifuge, and the pellet was resuspended in 1/20th of the original culture volume of TSS buffer (5 g PEG 8000, 1.5 mL of 1 M MgCl2, 2.5 mL of DMSO brought up to 50 mL total volume with LB medium and filter-sterilized). Approximately 100 ng of plasmid DNA was added to the resuspended cells, and after approximately 10 minutes incubation in an ice-water bath, cells were heat shocked at 42°C for 30 seconds and transferred to an ice water bath for 2 minutes. LB medium was added and cells were outgrown for ~1 hour before plating on LB plates containing 50 µg/mL kanamycin. Mutations were additionally made in

strain XQ52 by MAGE, as described previously, and p15SpeC was transformed as described above to generate an additional set of production strains.

15) Cell culturing for putrescine production

For putrescine production under batch conditions, cells were inoculated directly from colonies on fresh transformation plates into 300 µL of LB medium containing 50 µg/mL kanamycin in 96 well deepwell plates, and grown in a plate shaker overnight at 37°C with 300 rpm shaking. The following morning, cells were diluted 1:100 into a final volume of either 2.5 mL of R/2 medium (2 g/L (NH4)2HPO4, 6.75 g/L KH2PO4, 0.85 g/L citric acid, 0.7 g/L MgSO4·7H2O, 0.1 mM $CaCl_2$, trace elements as supplemented in M9 medium described previously, 10 g/L glucose, and 3 g/L (NH4)2SO4; pH adjusted to 6.80) containing 50 µg/mL kanamycin in 24 well MTP plates or 300 µL of R/2 medium in 96 well MTP plates. When strains were not lacI- (as in the XQ52 background), 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at inoculation to induce overexpression of SpeC on p15SpeC. Cultures were incubated in a plate shaker at 37°C with 300 rpm shaking, and samples were taken after 24 h and/or 48 h for analysis of putrescine production. One-tenth volume of 100% trichloroacetic acid and a final volume of 0.5 g/L HMDA was added as an internal standard. After vortexing and centrifugation, supernatants were stored at -20□C before further processing for polyamine analysis.

For semi-batch growth with periodic glucose/ammonia feeding, cells were inoculated directly from colonies on fresh transformation plates into 2.5 mL of LB medium containing 50 µg/mL kanamycin in 24 well deepwell plates, and grown in a plate shaker overnight at 37°C with 300 rpm shaking. The next morning, after withdrawing 100 µL for measuring OD600, the remaining culture volume was spun down in plates at 4000 rpm for 10 minutes, cell pellets were resuspended in 500 µL of R/2 medium (previously described), and cells were inoculated into 10.5 mL of R/2 medium containing 50 µg/mL kanamycin in Hamilton fermentors on a Hamilton VantageTM based cultivation robot. IPTG was added to lacI+ strains to a final concentration of 100 µM. A feed solution containing 500 g/L glucose, 154.5 g/L (NH4)2SO4, and 7.27 g/L MgSO4·7H2O (filter-sterilized) was fed into the fermentors. After 24 and 48 hours cultivation, 0.5 mL of cell culture was collected, 10 µL of 100 g/L HMDA was added as an internal standard, and 50 µL of 100% (w/v) trichloroacetic acid was added. Following vortexing and centrifugation, supernatants were stored at -20°C before further processing for polyamine analysis.

16) Derivatization and HPLC analysis of polyamines

50 µL of supernatants collected as described above were transferred to a glass tube containing 200 µL of 2 M NaOH. To this solution, 10 µL of 50% (v/v) benzoyl chloride in methanol was added to the tubes and they were immediately vortexed for 30 seconds to disperse the benzoyl chloride. The benzoylation reaction was allowed to proceed for 30 minutes, with vortexing approximately every 5 minutes. Benzoylated polyamines were then extracted into 1 mL of chloroform and 500 µL of the bottom chloroform layer was transferred to a new tube and evaporated to dryness under a nitrogen stream. To the dried residue in the tubes, 500 µL of 50% (v/v) acetonitrile in water was added. An external standard containing putrescine with 0.5 g/mL of HMDA as an internal standard was similarly prepared using the same procedure, and dilutions were made to enable the determination of a standard curve. 10 µL of each sample was injected on an Ultimate 3000 HPLC (Thermo Scientific) equipped with a Discovery® HS F5 column (2.1 x 150 mm, 3.0 µm particle size) (Supelco) with a UV detector (229 nm). The mobile phase consisted of 10 mM ammonium formate, pH 3 adjusted with formic acid (A) and acetonitrile (B), with the following linear gradients applied using a total flow rate of 0.5 mL/min: 10% B from 0 to 2 minutes, 10 to 45% B from 2 to 22 minutes, 45% B from 22 to 26 minutes, 45 to 10% B from 26 to 28 minutes, and 10% B from 28-30 minutes. Putrescine, cadaverine, HMDA, and excess benzoyl chloride appeared as peaks at retention times of 14.5, 15.9, 17.8, and 15.1 minutes, respectively.

17) Analysis of growth parameters (growth rate and lag time)

For data obtained with the Biolector microbioreactor system, self-baselined growth series were imported directly into a custom software platform that automatically detects growth phases and exports growth rates and lag times. In an earlier version of the software (values labeled in columns with "(1)", a line was fit to a detected linear region in semilog space to determine the growth rate. An updated version of the software (values labeled in columns with "(2)") implemented a direct exponential fit of a detected growth phase in linear space, resulting in higher weighting of the least squares fit to regions of the curve exhibiting higher growth. Additionally, the updated version of the software implemented an adaptive smoothing algorithm that split the data into variable sized windows that minimize the standard deviation of growth values within a time interval, and generated spline fits between points. Finally, the updated version of the software discarded regions where growth curves were fit but the signal-to-noise ratio was less than 1, to eliminate automatic detection of false growth phases. While automatic detection succeeded in detecting and fitting the dominant growth phase more than 95% of the time, all data was additionally manually curated to

ensure that the main growth phase was always selected and that false growth phases were not detected when growth was essentially absent.

For data obtained with the Growth Profiler, improved image analysis was additionally implemented to obtain the updated growth parameters. In the Tables below, for values labeled in columns with "(1)", integrated pixel values (which were later converted to OD_{600} using a calibration curve) were obtained directly from image analysis capabilities in the Growth Profiler software. In the Tables below, for values labeled in columns with "(2)", a new algorithm was implemented that automatically detected the pixel integration region in each well in each image by locating the darkest pixels in each well. These values were converted to OD_{600} with a calibration run in the same manner. The new algorithm provided for an improved accuracy in determining the growth rate, since it eliminated a slowly oscillating frequency that was sometimes observed in the original data, potentially related to the practical setup when scanning the plates.

Results

a) Wild-type tolerance to polyamines

The maximum measured concentration of putrescine at which K-12 MG1655 can grow was found to be 40 g/L (Table 3), with a nearly 26 hour lag time. Lag times and growth rates dropped steeply at concentrations above 30 g/L. At concentrations above 40 g/L, no growth was detected.

The maximum measured concentration of HMDA at which exponentially growing K-12 MG1655 can grow was found to be 40 g/L (Table 4). At concentrations above 40 g/L, there was a steep drop in growth, with zero growth detected at 50 g/L concentration.

Table 3. Growth of K-12 MG1655 in putrescine

	mean (1)		std. error (1)		mean (2)		std. error (2)	
putrescine (g/L)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0,926	1,3	0,178	0,1	0,651	0,5	0,026	0,2
10	0,698	1,4	0,086	0,0	0,538	1,0	0,015	0,1
20	0,577	2,2	0,020	0,2	0,392	1,5	0,011	0,1
30	0,350	3,4	0,057	0,7	0,317	6,2	0,007	0,3
40	0,108	12,3	0,041	3,0	0,148	25,8	0,016	5,8
50	0,023	19,8	0,040	-	0,000	-	0,000	-
75	0,000	-	0,000	-	0,000	-	0,000	-
100	0,000	-	0,000	-	0,000	-	0,000	-

Table 4. Growth of K-12 MG1655 in HMDA

	mean (1)		std. error (1)		mean (2)		std. error (2)	
HMDA (g/L)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0,946	1,4	0,032	0,0	0,698	0,8	0,005	0,1
10	0,806	1,3	0,059	0,1	0,605	0,8	0,021	0,1
20	0,601	1,4	0,047	0,1	0,394	0,3	0,005	0,1
30	0,475	1,7	0,056	0,2	0,241	0,0	0,005	0,0
40	0,216	2,6	0,026	0,2	0,136	2,3	0,003	0,7
50	0,000	-	0,000	-	0,000	-	0,000	-
75	0,000	-	0,000	-	0,000	-	0,000	-
100	0,000	-	0,000	-	0,000	-	0,000	-

Aiming for a starting growth rate of approximately 0.3 h-1, it was decided to begin evolutions at a concentration of 25 g/L putrescine and 25 g/L HMDA.

b) Resequencing of tolerant isolates

Variants detected in putrescine and HMDA evolved strains are presented in Tables 5 and 6, respectively. Each strain name corresponds to the chemical the strain was isolated from, the population the strain was isolated from, and the original number of the strain assigned during primary screening (*e.g.* PUTR3-1 is a putrescine-evolved strain isolated from population 3). In each table, strains are arranged such that all that were isolated from the same population are presented in the same rows. Strains with an asterisk (*) following their name are hypermutator strains, and only the mutation identified that can be associated with generating the hypermutator phenotype (here only in *mutS* or *mutT* in 2 HMDA populations) and those mutations that are shared with other mutations in the same gene in other strains are shown.

Table 5. Variants detected in putrescine-evolved isolates

coordinate	gene	change	coordinate	gene	change	coordinate	gene	change
PUTR2-4			**PUTR2-6**					
1907266	*cspC*	7 bp insertion (→CGTCCTG)	1907266	*cspC*	7 bp insertion (→CGTCCTG)			
3400986	*mreB*	N34K (A→C)	3400986	*mreB*	N34K (A→C)			
4186706	*rpoC*	V453I (G→A)	4186706	*rpoC*	V453I (G→A)			
PUTR3-1			**PUTR3-9**			**PUTR3-10**		
1211308	*mcrA/icdC*	noncoding SNP (G→T)	2678755	*yphF*	IS5 element insertion	1211308	*mcrA/icdC*	noncoding SNP (G→T)
1934806	*edd/zwf*	noncoding SNP (C→T)	2774803	*yfjW*	1 bp deletion	2661793	*iscR*	L113F (C→A)
2799867	*ygaC*	R43L (C→A)	3400453	*mreB*	E212A (T→G)	2799867	*ygaC*	R43L (C→A)
2804946	*proV*	1 bp insertion (→T)	3816611	*rph/yicC*	noncoding SNP (C→A)	2804946	*proV*	1 bp insertion (→T)
3816611	*rph/yicC*	noncoding SNP (C→A)	3823025	*spoT*	R209H (G→A)	2904286	*ygcE/queE*	122 bp deletion
3823025	*spoT*	R209H (G→A)	3911364	*pstS*	1 bp insertion (→T)	3816611	*rph/yicC*	noncoding SNP (C→A)
4178239	*nusG*	G166V (G→T)	4257602	*lexA*	N163I (A→T)	3823025	*spoT*	R209H (G→A)
4257602	*lexA*	N163I (A→T)				4178239	*nusG*	G166V (G→T)
						4257602	*lexA*	N163I (A→T)
						4267824	*tyrB*	L237F (A→C)
PUTR4-3			**PUTR4-7**			**PUTR4-8**		
457398	*clpP/clpX*	7 bp deletion	665554	*mrdB*	E254K (C→T)	665554	*mrdB*	E254K (C→T)
665554	*mrdB*	E254K (C→T)	962473	*rpsA*	D160V (A→T)	962473	*rpsA*	D160V (A→T)
1907410	*cspC*	IS5 element insertion	1907410	*cspC*	IS5 element insertion	1236007	*ycgB*	50 bp deletion
2805131	*proV*	1 bp insertion (→T)	2805131	*proV*	1 bp insertion (→T)	1907410	*cspC*	IS5 element insertion
4183154	*rpoB*	R637L (G→T)	4183154	*rpoB*	R637L (G→T)	2805131	*proV*	1 bp insertion (→T)
						4183154	*rpoB*	R637L (G→T)
						4392443	*glyV/glyX*	1 bp deletion
PUTR5-1			**PUTR5-6**			**PUTR5-8**		
568660	*emrE/ybcK*	noncoding SNP (C→T)	3214770	*rpoD*	E575A (A→C)	3214770	*rpoD*	E575A (A→C)
1755770	*pykF*	D25N (G→A)	4186551	*rpoC*	V401G (T→G)	4186551	*rpoC*	V401G (T→G)
2023551	*fliR*	IS5 element insertion	4452005	*ytfR*	noncoding SNP (G→A)			
3401016	*mreB*	I24M (A→C)						
3805049	*waaS*	1 bp deletion						
3823799	*spoT*	R467L (G→T)						
3910569	*pstS*	7 bp deletion						
4522146	*yjhG*	D650V (T→A)						

coordinate	gene	change	coordinate	gene	change	coordinate	gene	change
PUTR6-2			**PUTR6-7**			**PUTR6-10**		
2798597	*stpA/alaE*	IS1 element insertion	576891	*nmpC/essD*	noncoding SNP (C→T)	576891	*nmpC/essD*	noncoding SNP (C→T)
3336073	*murA*	G141A (C→G)	962933	*rpsA*	N313K (C→G)	777151	*tolA*	48 bp deletion
3377150	*sspA*	V91F (C→A)	1199680	*intE*	IS1 element insertion	962933	*rpsA*	N313K (C→G)
3899249	*yieK*	T49P (T→G)	1879829	*yeaR*	IS186 element insertion	1199680	*intE*	IS1 element insertion
3908805	*pstA*	2 bp deletion	1907448	*yobF*	IS5 element insertion	1879829	*yeaR*	IS186 element insertion
			2804858	*proV*	13 bp deletion	1907448	*yobF*	IS5 element insertion
			3079559	*cmtB/tktA*	noncoding SNP (C→T)	2804858	*proV*	13 bp deletion
			3197145	*glnE*	12 bp deletion	3079559	*cmtB/tktA*	noncoding SNP (C→T)
			3815859	*rph*	82 bp deletion	3267294	*tdcA/tdcR*	noncoding SNP (G→T)
			4186186	*rpoC*	noncoding SNP (G→C)	3815859	*rph*	82 bp deletion
						4282760	*yjcF*	R106G (T→C)
PUTR7-1			**PUTR7-7**			**PUTR7-9**		
962922	*rpsA*	D310Y (G→T)	3214770	*rpoD*	E575A (A→C)	1673532	*mdtJ/tqsA*	181 bp deletion
3316916	*nusA*	M204R (A→C)	3335317	*murA*	Y393S (T→G)	3214770	*rpoD*	E575A (A→C)
3400811	*mreB*	H93N (G→T)	4183154	*rpoB*	R637L (G→T)	3335317	*murA*	Y393S (T→G)
3823799	*spoT*	R467H (G→A)				4183154	*rpoB*	R637L (G→T)
PUTR8-3			**PUTR8-6**			**PUTR8-10**		
2807247	*proX*	1 bp insertion (→T)	83670	*leuL*	3 bp deletion	562667	*sfmH*	F11S (T→C)
3318960	*argG*	noncoding SNP (C→A)	701231	*nagC*	1 bp deletion	700785	*nagC*	47 bp deletion
3400195	*mreB*	A298V (G→A)	2025435	*yodD/yedP*	noncoding SNP (T→A)	2807247	*proX*	1 bp insertion (→T)
3473612	*rpsG*	L157* (A→C)	2807247	*proX*	1 bp insertion (→T)	3318960	*argG*	noncoding SNP (C→A)
3815801	*pyrE/rph*	1 bp deletion	3318960	*argG*	noncoding SNP (C→A)	3400195	*mreB*	A298V (G→A)
3823811	*spoT*	R471H (G→A)	3400195	*mreB*	A298V (G→A)	3473612	*rpsG*	L157* (A→C)
			3473612	*rpsG*	L157* (A→C)	3815801	*pyrE/rph*	1 bp deletion
			3815801	*pyrE/rph*	1 bp deletion	3823811	*spoT*	R471H (G→A)
			3823811	*spoT*	R471H (G→A)			

Table 6. Variants detected in HMDA-evolved isolates

coordinate	gene	change	coordinate	gene	change	coordinate	gene	change
HMDA1-10								
962939	*rpsA*	N315K (C→A)						
2694102	*purL*	C481F (C→A)						
2804858	*proV*	13 bp deletion						
3816611	*rph/yicC*	noncoding SNP (C→A)						
3823025	*spoT*	R209H (G→A)						
4181786	*rpoB*	G181V (G→T)						
4257602	*lexA*	N163I (A→T)						
HMDA2-1			**HMDA2-8**					
963273	*rpsA*	3 bp substitution (→CGT)	963273	*rpsA*	3 bp substitution (→CGT)			
2804836	*proV*	IS1 element insertion	2804836	*proV*	IS1 element insertion			
2968160	*ptsP*	1 bp deletion	2968160	*ptsP*	1 bp deletion			
3815801	*pyrE/rph*	1 bp deletion	3815801	*pyrE/rph*	1 bp deletion			
HMDA3-4			**HMDA3-5**			**HMDA3-6**		
702592	*nagA*	1 bp insertion (→G)	691772	*ybeX*	12 bp deletion	702592	*nagA*	1 bp insertion (→G)
2798606	*alaE*[*proW*]	7565 bp deletion	701405	*nagC*	E64* (C→A)	2177307	*gatY/fbaB*	IS1 element insertion
3815809	*pyrE/rph*	1 bp deletion	2798606	*alaE*[*proW*]	7565 bp deletion	2798606	*alaE*[*proW*]	7565 bp deletion
3933122	*kup*	P603T (C→A)	2879763	*ygbT*	L4F (G→A)	3815809	*pyrE/rph*	1 bp deletion
4188767	*rpoC*	R1140C (C→T)	2991218	*ygeF/ygeG*	noncoding SNP (A→G)	3933122	*kup*	P603T (C→A)
			3815809	*pyrE/rph*	1 bp deletion	4188767	*rpoC*	R1140C (C→T)
			3933122	*kup*	P603T (C→A)			
HMDA4-2*			**HMDA4-6***			**HMDA4-9***		
962923	*rpsA*	D310G (A→G)	962923	*rpsA*	D310G (A→G)	962923	*rpsA*	D310G (A→G)
1729289	*lhr*	R68Q (G→A)	2805832	*proV*	IS1 element insertion	2805832	*proV*	IS1 element insertion
2805832	*proV*	IS1 element insertion	2859212	*mutS*	6 bp insertion (→GGCGTG)	2859212	*mutS*	6 bp insertion (→GGCGTG)
2859212	*mutS*	6 bp insertion (→GGCGTG)	3815859	*rph*	82 bp deletion	3815859	*rph*	82 bp deletion
3815859	*rph*	82 bp deletion	3823861	*spoT*	R488C (C→T)	3823861	*spoT*	R488C (C→T)
3823861	*spoT*	R488C (C→T)	4185708	*rpoC*	L120P (T→C)	4185708	*rpoC*	L120P (T→C)
4181706	*rpoB*	noncoding SNP (C→T)						
4185708	*rpoC*	L120P (T→C)						

coordinate	gene	change	coordinate	gene	change	coordinate	gene	change
HMDA5-4			**HMDA5-5**			**HMDA5-10**		
700602	*nagC*	IS1 element insertion	691321	*ybeX*	L155Q (A→T)	2798597	*stpA/alaE*	IS1 element insertion
2798606	*alaE* [*proV*]	7067 bp deletion	700602	*nagC*	IS1 element insertion	2966573	*ptsP*	D621A (T→G)
2966573	*ptsP*	D621A (T→G)	2798606	*alaE* [*proV*]	7067 bp deletion	3815809	*pyrE/rph*	1 bp deletion
3310266	*pnp*	E301D (C→A)	2966573	*ptsP*	D621A (T→G)	3908248	*pstB*	A40E (G→T)
3815809	*pyrE/rph*	1 bp deletion	3815809	*pyrE/rph*	1 bp deletion	4485639	*pepA*	S105A (A→C)
4378331	*ampC*	I205T (A→C)						
HMDA6-3*			**HMDA6-7***					
111300	*mutT*	L86P (T→C)	111300	*mutT*	L86P (T→C)			
701839	*nagA*	T305P (T→G)	701839	*nagA*	T305P (T→G)			
2804831	*proV*	7 bp deletion	2804831	*proV*	7 bp deletion			
2879197	*ygbT*	E192D (T→G)	2879197	*ygbT*	E192D (T→G)			
3815859	*rph*	82 bp deletion	3104042	*mutY*	L344V (T→G)			
3823987	*spoT*	G530C (G→T)	3815859	*rph*	82 bp deletion			
			3823987	*spoT*	G530C (G→T)			
HMDA7-1			**HMDA7-7**			**HMDA7-10**		
2104070	*wbbK*	1 bp deletion	3317072	*nusA*	L152R (A→C)	2104070	*wbbK*	1 bp deletion
2818240	*argY/argV*	284 bp deletion	3377173	*sspA*	F83C (A→C)	2818220	*argY/argV*	271 bp deletion
3317072	*nusA*	L152R (A→C)	3473612	*rpsG*	L157* (A→C)	3317072	*nusA*	L152R (A→C)
3377173	*sspA*	F83C (A→C)				3377173	*sspA*	F83C (A→C)
3473612	*rpsG*	L157* (A→C)				3473612	*rpsG*	L157* (A→C)
HMDA8-5			**HMDA8-9**			**HMDA8-10**		
358399	*cynR*	T98P (T→G)	700980	*nagC*	Y205* (G→C)	700980	*nagC*	Y205* (G→C)
700980	*nagC*	Y205* (G→C)	1728512	*rnt*	T56P (A→C)	1728512	*rnt*	T56P (A→C)
1728512	*rnt*	T56P (A→C)	1732811	*lhr*	S1242I (G→T)	1732811	*lhr*	S1242I (G→T)
1732811	*lhr*	S1242I (G→T)	1744313	*mdtK*	V286E (T→A)	1744313	*mdtK*	V286E (T→A)
1744313	*mdtK*	V286E (T→A)	2804858	*proV*	13 bp deletion	2804858	*proV*	13 bp deletion
2522653	*xapR/xapB*	noncoding SNP (G→A)	3815801	*pyrE/rph*	1 bp deletion	3815801	*pyrE/rph*	1 bp deletion
2804858	*proV*	13 bp deletion				4457112	*mpl*	4 bp deletion
3815801	*pyrE/rph*	1 bp deletion						

c) Characterization of selected isolates

Each re-sequenced isolate was characterized using the Biolector system for growth at the screening concentration of chemical (38 g/L putrescine or HMDA) in biological triplicates. The average growth rate and lag time for the three replicates are shown in Tables 7 (putrescine) and 8 (HMDA), indicating standard deviation about the mean for the measurement at each time point, representing the growth characteristics of the wild-type K-12 MG1655 and the isolates from each population.

Large differences in growth behavior amongst evolved isolates can be noted. Better growing strains are defined by both the higher growth rate and the reduced lag time (*i.e.*, at what time the cultures begin growing). Some isolates exhibit poor performance (*e.g.* PUTR5-1 and HMDA8-10). The phenotype to genotype relationship infers mutations that are of highest interest and those that are not of interest. Another example of this would, for example, be when two strains are growing identically (*e.g.* HMDA3-4 and HMDA3-6). This indicates that any differences in mutations between these two isolates are not important for tolerance. For HMDA3-4 and HMDA3-6, this suggests that the intergenic mutation between *gatY* and *fbaB* does not contribute to the tolerance phenotype.

Table 7 - Averaged growth data of biological triplicates of putrescine-evolved isolates grown in M9 + 1% glucose + 38 g/L putrescine

	mean (1)		std. error (1)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,100	12,0	0,003	5,6	0,112	10,4	0,044	9,3
PUTR2-4	0,224	25,0	0,011	2,6	0,212	25,6	0,021	2,6
PUTR2-6	0,232	21,6	0,014	2,4	0,237	23,3	0,016	1,6
PUTR3-1	0,326	14,4	0,008	1,7	0,370	16,1	0,010	1,7
PUTR3-9	0,181	14,6	0,049	10,6	0,197	19,3	0,048	3,9
PUTR3-10	0,245	22,8	0,107	11,2	0,308	24,9	0,080	11,3
PUTR4-3	0,283	8,0	0,061	1,7	0,272	10,5	0,032	2,6
PUTR4-7	0,287	14,5	0,019	1,5	0,259	17,1	0,014	2,5
PUTR4-8	0,237	17,4	0,034	2,3	0,244	25,7	0,018	9,0
PUTR5-1	0,130	27,5	0,035	3,9	0,167	28,1	0,040	3,0
PUTR5-6	0,202	10,5	0,010	1,8	0,223	11,2	0,003	1,4
PUTR5-8	0,199	8,7	0,002	2,9	0,214	10,9	0,008	0,7
PUTR6-2	0,192	19,0	0,021	5,5	0,234	23,0	0,022	7,8
PUTR6-7	0,076	21,7	0,003	23,8	0,356	42,4	0,162	5,7
PUTR6-10	0,242	10,6	0,019	0,6	0,288	19,2	0,012	1,2

PUTR7-1	0,267	9,4	0,019	0,5	0,242	12,5	0,029	0,6
PUTR7-7	0,267	3,5	0,042	0,4	0,282	12,3	0,021	0,9
PUTR7-9	0,277	5,3	0,016	0,8	0,265	11,4	0,014	0,8
PUTR8-3	0,236	24,1	0,041	2,1	0,274	24,2	0,028	4,3
PUTR8-6	0,284	9,7	0,020	2,1	0,342	11,6	0,039	0,2
PUTR8-10	0,285	6,6	0,020	1,9	0,335	11,1	0,026	0,2

Table 8 - Averaged growth data of biological triplicates of HMDA-evolved isolates grown in M9 + 1% glucose + 38 g/L HMDA

	mean (1)		std. error (1)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,048	38,2	0,068	13,9	0,051	29,5	0,004	1,6
HMDA1-10	0,314	11,6	0,022	0,8	0,343	12,3	0,013	0,6
HMDA2-1	0,266	32,8	0,014	2,0	0,324	33,3	0,011	3,1
HMDA2-8	0,251	29,5	0,034	5,4	0,338	30,9	0,022	3,8
HMDA3-4	0,428	18,7	0,096	1,8	0,335	18,4	0,052	2,0
HMDA3-5	0,508	13,0	0,035	1,0	0,649	14,4	0,041	0,9
HMDA3-6	0,443	19,7	0,047	1,7	0,381	19,7	0,074	2,1
HMDA4-2	0,380	15,2	0,034	9,7	0,407	18,5	0,062	9,0
HMDA4-6	0,322	22,6	0,008	9,6	0,355	24,8	0,015	5,3
HMDA4-9	0,298	9,7	0,003	1,7	0,317	11,8	0,018	2,8
HMDA5-4	0,488	27,6	0,042	4,3	0,414	27,4	0,066	3,4
HMDA5-5	0,393	32,6	0,003	1,7	0,390	34,4	0,078	1,4
HMDA5-10	0,250	26,3	0,001	2,8	0,278	32,8	0,049	8,6
HMDA6-3	0,341	14,3	0,006	2,2	0,398	15,3	0,028	1,1
HMDA6-7	0,320	12,4	0,036	2,1	0,382	14,9	0,025	0,9
HMDA7-1	0,490	5,9	0,010	0,2	0,387	6,9	0,012	0,9
HMDA7-7	0,353	11,8	0,068	4,1	0,342	13,0	0,043	2,8
HMDA7-10	0,424	7,2	0,041	1,0	0,348	7,4	0,018	1,1
HMDA8-5	0,212	24,3	0,145	11,7	0,245	23,2	0,126	8,2
HMDA8-9	0,176	8,5	0,249	3,9	0,197	16,8	0,140	13,8
HMDA8-10	0,206	32,6	0,049	3,9	0,229	30,8	0,068	5,4

d) Sole carbon source plate growth assay

Wild-type, putrescine, and HMDA evolved strains were struck on M9 agar containing putrescine or HMDA as a sole carbon source. No growth was observed on HMDA plates indicating that *E. coli* cannot utilize HMDA as a sole carbon source. *E. coli* is known to be able to degrade putrescine as a sole carbon source, and slow growth was observed on putrescine containing plates. After a few weeks, widely varying growth trends could be observed

between strains (Table 9), which can be correlated with mutation profiles. K-12 MG1655 exhibited the most robust growth on plates, together with PUTR4-3, PUTR5-6, PUTR5-8, and PUTR6-2. Strains that possess losses-of-function in ProV or ProX are indicated in Table 9, thus it is notable that 4 out of 5 of the best growing strains still possess functional ProVWX. This is suggestive of ProVWX, an ABC transporter having known promiscuous quaternary amine import properties, being involved in putrescine import.

PUTR2-4, PUTR2-6, PUTR5-1, PUTR7-1, PUTR7-7, and PUTR7-9 possess intact ProVWX however they still exhibit impaired growth. All of these strains also possess coding mutations in *mreB* or *murA*, indicating that these genes, possibly related to changes in cell shape (see the in a later section "Flow cytometric analysis of cell morphology"), are also resulting in diminished import or catabolism of putrescine. A marked difference in ability to grow on putrescine as a sole carbon source can also be observed between PUTR8-3, which exhibits moderate growth, and PUTR8-6 and PUTR8-10 which exhibit nearly completely abolished growth. These strains have similar sets of mutations, with PUTR8-3 lacking only the frameshift mutation in *nagC*. NagC is a transcriptional regulator that binds N-acetylglucosamine 6-phosphate, a precursor for peptidoglycan biosynthesis, and controls the expression of genes to coordinate the biosynthesis and degradation of this component. Thus cells lacking functional NagC may also possess cell wall modifications that reduce the import or catabolism of putrescine.

A mutational correlation analysis was additionally performed by assigning a qualitative growth defect score between 1 and 10 to each strain and determining the correlation coefficient for each mutation assuming a linear model for their impact on the growth phenotype. When this is performed by minimizing the sum of the square of the residuals between the calculated and assigned values for the growth defect score, the mutated gene with the highest correlation coefficient is *mreB* (6.52), followed by *rpoB* (3.09), *rpoD* (2.26), and *nagC* (1.89), suggesting that these genes were causative for the associated growth phenotypes on putrescine as a sole carbon source. Mutations in *proV, proX*, or *proW* were found to be non-causative for growth on putrescine as a sole carbon source in this fitted model (correlation coefficient of zero), which does not account for possible genetic interactions. Thus mutations in *proV, proX*, or *proW* are likely not involved in the direct import of putrescine and mutations in MreB are likely involved in reducing intracellular levels of putrescine.

Table 9 - Growth of sequenced putrescine-evolved isolates on M9 agar plates containing putrescine as a sole carbon source

	growth score	proVWX mutation	nagC/nagA mutation	mreB/murA mutation
MG1655	++++	no	no	no
PUTR2-4	+	no	no	yes
PUTR2-6	+	no	no	yes
PUTR3-1	+	yes	no	no
PUTR3-9	+	no	no	yes
PUTR3-10	+	yes	no	no
PUTR4-3	+++	yes	no	no
PUTR4-7	+	yes	no	no
PUTR4-8	+	yes	no	no
PUTR5-1	+	no	no	yes
PUTR5-6	+++	no	no	no
PUTR5-8	+++	no	no	no
PUTR6-2	++++	no	no	no
PUTR6-7	++	yes	no	no
PUTR6-10	++	yes	no	no
PUTR7-1	+	no	no	yes
PUTR7-7	+	no	no	yes
PUTR7-9	+	no	no	yes
PUTR8-3	++	yes	no	yes
PUTR8-6	none	yes	yes	yes
PUTR8-10	none	yes	yes	yes

e) Knockout strain growth performance – high putrescine concentrations

Group 1 (probable loss-of-function) mutations were identified from re-sequencing results as described in methods. Two different frameshift mutations were present in proV and one frameshift mutation was present in *proX* in populations 3, 4, 6, and 8, respectively (*proV* and *proX* encode different subunits of the same protein). Frameshift mutations and insertion sequence elements were identified in *cspC* in populations 2 and 4, and an insertion sequence element was identified in population 6 in *yobF*, a protein of unknown function found in the same operon as *cspC*. Two different frameshift deletions were identified in *nagC* in population 8. Insertion sequence elements were identified in *yeaR* in population 6. Two different frameshift mutations were identified in individual isolates in populations 3 and 5. Any additional mutations tested for imparting putrescine tolerance were identified in HMDA-evolved strains (description follows) and were also tested in putrescine due to the similarity

of the two chemicals and similar sets of genes being mutated following evolution. Combinations of mutations were selected partly based on the presence of particular mutations with each other, so some gene disruptions were not tested alone (*e.g. nagC*).

Initially, single knockouts and a few double knockout combinations were screened with the Growth Profiler at two concentrations: 19 g/L and 38 g/L putrescine. Growth data for individual biological replicates are shown in Table 10.

In this testing format, it was found that of the knockouts tested, deletion of *proV* significantly increased the growth rate at 19 g/L and decreased the lag time in 38 g/L. Double knockouts, which all contained a deletion of *proV* and another gene, did not appear to exhibit improved growth relative to the *proV* deletion alone.

Strains including additional double and triple knockout strains that had been constructed based on both these Growth Profiler results and mutations found in HMDA evolved strains (see data below) were then tested in the Biolector testing format together with a selection of evolved strains (Table 11).

The best performing strains were the *proV* single deletion strain and the *proV cspC* double deletion strain. Using the original algorithm (see section 17), the *proV yobF* double-deletion strain was possible also among them (although significant variation between replicates). It should be noted that *cspC* and *yobF* are transcribed in the same operon, so there is a possibility that disruption of one affects expression of the other, and/or that they are related in function and are possibly even involved in the same overall cellular response.

A second Biolector experiment was performed repeating growth of several of the strains shown in the first experiment but also including a few additional strains (Table 12).

The best performing strain in terms of lag time in this run was the *cspC* single knockout, while the strain with the highest growth rate but non-improved lag time was the *ptsP* single knockout. Reduced lag times were also apparent in the *proV* single knockout, *proV cspC* double knockout, *proV ptsP* double knockout, and *proV ptsP wbbK* triple knockout strains.

The Keio collection of gene knockouts is a commercial collection of knockouts in nearly all non-essential genes and ORFs in *E. coli* strain BW25113. This strain is a K-12 derivative and possesses known mutations relative to the K-12 MG1655 background. All Keio collection strains with knockouts in genes that were found to be mutated in Table 5, minus knockouts in genes that were already screened in the K-12 MG1655 background in Tables 10, 11, and 12,

were screened for growth against the BW25113 control in M9 + 1% glucose + 32 g/L and also plus 38 g/L putrescine in the Growth Profiler screening format. Averaged growth data for 3 biological replicate cultures were calculated for each strain at 32 g/L and 38 g/L (Table 13). No significant improvements in growth rate or reductions in lag time were observed.

A list of all gene disruption mutants in both the K-12 MG1655 and BW25113 background strains that exhibited increased tolerance to putrescine is shown in Table 14.

Table 10 - Preliminary screening of predicted loss-of-function mutations found in evolved strains in different concentrations of putrescine

	19 g/L putrescine				38 g/L putrescine			
	mean (1)		std. error (1)		mean (1)		std. error (1)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,528	2,3	0,045	0,6	0,244	6,6	0,008	1,4
MG1655 proV::kan	0,586	2,1	0,027	0,3	0,197	5,9	0,011	5,0
MG1655 cspC::kan	0,531	1,9	0,056	0,7	0,125	16,6	0,007	2,2
MG1655 yeaR::kan	0,535	2,2	0,020	0,4	0,212	4,5	0,015	0,8
MG1655 pstS::kan	0,456	3,2	0,024	1,7	0,176	10,2	0,033	0,1
MG1655 ΔproV cspC::kan	0,613	2,0	0,040	0,6	0,151	21,0	0,005	6,3
MG1655 ΔproV yobF::kan	0,562	1,9	0,010	0,3	0,211	9,8	0,011	4,2
MG1655 ΔproV nagC::kan	0,522	1,8	0,008	0,6	0,186	6,6	0,022	5,3
	mean (2)		std. error (2)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,457	7,9	0,015	0,2	0,172	22,6	0,009	0,4
MG1655 proV::kan	0,535	7,0	0,009	0,2	0,161	21,1	0,012	0,3
MG1655 cspC::kan	0,453	7,7	0,011	0,1	0,157	38,6	0,077	6,8
MG1655 yeaR::kan	0,459	8,1	0,011	0,0	0,156	23,6	0,003	0,5
MG1655 pstS::kan	0,322	8,9	0,010	0,5	0,085	26,3	0,024	1,9
MG1655 ΔproV cspC::kan	0,533	7,1	0,008	0,1	0,142	34,5	0,005	1,3
MG1655 ΔproV yobF::kan	0,534	7,2	0,005	0,0	0,175	25,5	0,009	0,1
MG1655 ΔproV nagC::kan	0,456	7,8	0,006	0,3	0,123	25,8	0,028	3,0

Table 11 - First Biolector growth screen of loss-of-function mutants inferred from putrescine evolved isolates

	mean (1)		std. error (1)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,110	17,6	0,002	1,2	0,105	18,9	0,020	3,5

PUTR3-1	0,374	11,8	0,013	0,5	0,372	13,2	0,014	0,3
PUTR4-3	0,335	6,7	0,039	0,8	0,267	9,0	0,005	0,2
PUTR5-6	0,221	5,6	0,012	0,0	0,240	9,2	0,000	0,4
PUTR7-7	0,316	-	0,024	-	0,332	9,1	0,011	1,4
PUTR8-10	0,318	5,6	0,003	0,7	0,340	19,0	0,045	18,0
MG1655 cspC::kan	0,133	20,5	0,010	1,4	0,103	14,6	0,013	1,5
MG1655 proV::kan	0,105	24,4	0,008	2,8	0,127	28,7	0,017	3,2
MG1655 yeaR::kan	0,071	24,2	-	-	0,078	31,0	0,017	26,1
MG1655 pstS::kan	0,102	14,9	0,009	3,1	0,068	17,4	0,009	1,2
MG1655 ΔproV cspC::kan	0,147	21,6	0,021	1,6	0,121	27,7	0,060	5,9
MG1655 ΔproV yobF::kan	0,132	20,5	0,039	0,1	0,105	27,2	0,054	11,6
MG1655 ΔproV nagC::kan	0,135	17,3	0,018	2,4	0,094	26,5	0,053	2,6
MG1655 ΔproV wbbK::kan	0,112	17,0	0,031	1,1	0,115	18,0	0,023	7,2
MG1655 ΔproV ptsP::kan	0,100	27,8	0,021	1,7	0,088	32,8	0,026	2,8

Table 12 - Second Biolector growth screen of loss-of-function mutants inferred from putrescine evolved isolates

Table 12A:

	mean (1)		std. error (1)		mean (phase 2) (1)		std. error (phase 2) (1)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ_2 (h^{-1})	$t_{lag,2}$ (h)	μ_2 (h^{-1})	$t_{lag,2}$ (h)
MG1655	0,118	39,9	0,016	0,6	0,121	14,5	0,016	1,5
PUTR3-1	0,400	10,0	0,019	0,6	-	-	-	-
PUTR4-3	0,351	4,6	0,040	1,1	-	-	-	-
PUTR5-6	0,205	1,9	0,011	0,5	-	-	-	-
PUTR7-7	0,312	2,0	0,055	1,8	-	-	-	-
PUTR8-10	0,304	4,8	0,012	1,6	-	-	-	-
MG1655 proV::kan	0,136	20,7	0,008	1,8	-	-	-	-
MG1655 cspC::kan	0,160	17,3	0,008	1,1	-	-	-	-
MG1655 ptsP::kan	0,177	29,6	0,023	3,9	-	-	-	-
MG1655 wbbK::kan	0,099	38,9	0,011	3,1	0,101	12,6	0,029	4,7
MG1655 ΔproV cspC::kan	0,149	24,8	0,004	5,2	-	-	-	-
MG1655 ΔproV nagC::kan	0,119	38,6	0,019	3,0	0,143	10,2	0,000	0,2
MG1655 ΔproV ptsP::kan	0,139	20,1	0,042	9,2	-	-	-	-
MG1655 ΔproV wbbK::kan	0,111	29,3	0,034	12,0	0,128	14,2	0,002	0,8
MG1655 ΔproV ΔptsP wbbK::kan	0,151	22,3	0,008	1,8	-	-	-	-
MG1655 ΔproV ΔptsP nagC::kan	0,152	35,1	0,008	7,7	0,114	16,6	0,036	3,4

Table 12B:

strain	mean (2)		std. error (2)	
	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,171	32,5	0,024	1,9
PUTR3-1	0,390	13,1	0,016	0,2
PUTR4-3	0,261	7,1	0,002	0,2
PUTR5-6	0,215	6,7	0,004	0,3
PUTR7-7	0,297	8,7	0,021	0,3
PUTR8-10	0,354	8,6	0,007	0,2
MG1655 proV::kan	0,145	24,9	0,009	1,5
MG1655 cspC::kan	0,125	14,8	0,003	0,6
MG1655 ptsP::kan	0,208	31,4	0,082	5,0
MG1655 wbbK::kan	0,131	32,1	0,013	4,1
MG1655 ΔproV cspC::kan	0,143	24,4	0,021	7,0
MG1655 ΔproV nagC::kan	0,170	35,7	0,018	1,5
MG1655 ΔproV ptsP::kan	0,152	25,5	0,014	3,8
MG1655 ΔproV wbbK::kan	0,132	19,7	0,041	10,8
MG1655 ΔproV ΔptsP wbbK::kan	0,140	22,4	0,006	1,9
MG1655 ΔproV ΔptsP nagC::kan	0,174	34,3	0,030	2,4

Table 13 - Keio collection mutants exhibiting qualitatively improved growth in Growth Profiler screening with 32 g/L and 38 g/L putrescine

	32 g/L putrescine				38 g/L putrescine			
	mean (1)		std. error (1)		mean (1)		std. error (1)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
BW25113	0,336	3,9	0,002	1,4	0,248	8,0	0,002	1,3
BW25113 yjhG::kan	0,245	5,8	0,037	0,5	-	-	-	-
BW25113 ygcE::kan	0,284	3,8	0,021	2,7	0,167	12,9	0,026	9,6
BW25113 waaS::kan	-	-	-	-	0,280	7,7	0,018	1,5
BW25113 rpoD::kan	0,296	3,4	0,002	1,1	0,238	5,1	0,018	2,4
BW25113 sfmH::kan	0,310	3,3	0,010	2,7	0,179	4,2	0,027	5,0
BW25113 tdcR::kan	0,352	2,4	0,027	1,1	0,214	7,0	0,014	0,6
BW25113 proX::kan	0,303	2,8	-	-	0,285	8,3	0,039	1,2
BW25113 yobF::kan	0,339	3,2	0,030	0,9	0,226	8,1	0,075	2,6
BW25113 ytfR::kan	0,289	4,1	0,006	1,2	0,192	9,8	0,032	2,4
BW25113 rph::kan	0,311	2,3	0,061	1,0	0,205	6,5	0,023	4,2
BW25113 mdtJ::kan	0,288	3,6	0,002	0,6	0,276	5,9	0,007	1,9
BW25113 yicC::kan	0,375	5,1	0,037	5,0	0,286	8,7	0,007	0,9

BW25113 essD::kan	0,241	5,0	0,067	1,3	0,257	11,6	-	-
BW25113 yjcF::kan	0,301	3,5	0,032	0,7	0,225	7,0	0,032	1,4
BW25113 iscR::kan	0,295	3,6	0,006	0,4	0,195	6,2	0,018	0,3
BW25113 yedP::kan	0,337	5,0	0,039	0,5	0,234	5,4	0,038	1,8
	mean (2)		std. error (2)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
BW25113	0,308	12,9	0,012	0,1	0,198	22,6	0,029	1,2
BW25113 yjhG::kan	0,209	18,7	0,023	1,1	0,107	35,6	0,052	10,3
BW25113 ygcE::kan	0,276	13,8	0,026	1,0	0,180	22,6	0,026	1,4
BW25113 waaS::kan	0,259	13,9	0,013	0,2	0,181	23,3	0,004	0,2
BW25113 rpoD::kan	0,246	23,9	0,086	18,7	0,209	21,1	0,010	0,1
BW25113 sfmH::kan	0,285	13,9	0,072	3,2	0,185	23,5	0,049	6,5
BW25113 tdcR::kan	0,270	13,6	0,010	0,1	0,196	21,2	0,009	0,4
BW25113 proX::kan	0,280	13,4	0,025	0,6	0,162	22,9	0,024	0,5
BW25113 yobF::kan	0,293	13,6	0,009	0,3	0,173	22,4	0,006	1,0
BW25113 ytfR::kan	0,286	13,6	0,009	0,2	0,187	22,1	0,019	0,5
BW25113 rph::kan	0,289	13,8	0,003	0,1	0,157	23,9	0,002	0,5
BW25113 mdtJ::kan	0,256	15,6	0,029	3,1	0,139	33,3	0,048	14,3
BW25113 yicC::kan	0,299	13,4	0,007	0,7	0,199	22,4	0,022	1,4
BW25113 essD::kan	0,262	14,4	0,026	0,6	0,188	22,9	0,008	0,4
BW25113 yjcF::kan	0,215	19,1	0,031	1,2	0,141	32,3	0,016	2,5
BW25113 iscR::kan	0,301	13,4	0,002	0,3	0,192	22,9	0,015	1,0
BW25113 yedP::kan	0,263	14,5	0,014	0,5	0,155	28,5	0,008	0,4

Table 14: Gene deletion mutants exhibiting improved growth in high concentrations of putrescine

Strain genotype	Improved growth parameter
K-12 MG1655 *proV::kan*	Growth rate and/or lag time
K-12 MG1655 *cspC::kan*	Lag time
K-12 MG1655 *ptsP::kan*	Growth rate
K-12 MG1655 Δ*proV wbbK::kan*	Lag time
K-12 MG1655 Δ*proV cspC::kan*	Growth rate and/or lag time
K-12 MG1655 Δ*proV yobF::kan*	Growth rate
K-12 MG1655 Δ*proV ptsP::kan*	Lag time
K-12 MG1655 Δ*proV* Δ*ptsP wbbK::kan*	Llag time

The strains in Table 15 are a list of those tested but that were assessed to offer no significant improvement in growth in high concentrations of putrescine.

Table 15: Gene deletion mutants that were tested but did not improve growth in high concentrations of putrescine

Strain genotype	Growth rate improvement	Lag time improvement
K-12 MG1655 *yeaR::kan*	none	none
K-12 MG1655 *pstS::kan*	none	none
K-12 MG1655 *wbbK::kan*	none	none
K-12 MG1655 Δ*proV nagC::kan*	negative	negative
K-12 MG1655 Δ*proV* Δ*ptsP nagC::kan*	negative	negative
All available Keio collection strains with knockouts in individual genes shown in Table 5, except for those listed in Table 14	none	none

f) Knockout strain performance - HMDA

Two different frameshift mutations and two different insertion sequence elements in *proV* were identified in populations 1, 2, 4, 6, and 8. Another large deletion spanning *proV* and part of *proW* was also identified in population 3. Insertion sequence elements and SNPs generating premature stop codons in *nagC* were present in isolates from populations 3 and 5, and all isolates from population 8. A frameshift mutation and coding SNP were identified in *nagA* in populations 3 (the isolates that did not have the *nagC* mutation) and 6. One frameshift mutation and one coding SNP were identified in *ptsP* in populations 2 and 5. A frameshift mutation in *wbbK* was found in population 7. Any additional mutations tested for imparting HMDA tolerance were identified in putrescine-evolved strains (description follows) and were also tested in HMDA due to the similarity of the two chemicals and similar sets of genes being mutated following evolution. A *nagA* deletion mutant was not tested due to previous work in our lab showing that this deletion mutant behaves very similarly to the *nagC* deletion mutant, with both genes involved in the same pathway. We previously isolated transposon insertion mutants of *nagC* and *nagA* from a library in *E. coli* W following selection on 0.6 M NaCl and confirmed improved growth of clean deletion mutants in that condition (Lennen and Herrgård, Appl. Environ. Microbiol., 2014).

Initially, single knockouts and a few double knockout combinations were screened in the Growth Profiler at two concentrations: 19 g/L and 38 g/L HMDA. Growth data are shown in Table 16.

In this testing format, all strains shown in Table 16 exhibited improved growth at 19 g/L, with increased growth rates and equivalent or reduced lag times. At 38 g/L, only the *proV* and *ptsP* single knockout strains, and *proV*+*ptsP* double knockout strains exhibited improved

growth, with both increased growth rates and decreased lag times. The *proV nagC* double knockout strain notably exhibited completely abolished growth in 38 g/L HMDA. The *proV+ptsP* double knockout exhibited a much higher growth rate than other strains.

Based on the results from this first run in the Growth Profiler, some additional combination knockout strains were constructed and tested in the same format. The growth data for 38 g/L based on the averaged of three biological replicates with the standard deviation between values at each timepoint are shown in Table 17.

It was observed that of the strains tested, K-12 *ΔproV ptsP::kan* remained the best growing strain. The *proV wbbK* double knockout strain exhibited a slight improvement in growth rate and reduction in lag time compared to the *proV* single knockout.

Strains including an additional triple knockout combination based on the Growth Profiler results were then tested in the Biolector testing format in two separate experiments together with a selection of evolved strains (Table 18).

Greatly improved growth over the wild-type was observed for the *proV* and *ptsP* single deletion mutants, with moderately improved growth for the *wbbK* single deletion mutant. The *proV wbbK* double mutant performed worse than the *proV* single mutant, however the *proV ptsP wbbK* triple mutant performed both better than both combinations of double mutants. Due to some large variations between replicates for many strains, the strain genotypes were confirmed by colony PCR and the experiment was also repeated again on another date (Table 19).

Again, the *proV* and *ptsP* single mutants exhibited significantly improved growth compared to the wild-type, with the *wbbK* mutant exhibiting a small improvement. The *proV ptsP* and *proV ptsP wbbK* double and triple mutants performed better than the *proV wbbK* double deletion strain, which also performed significantly worse than the *proV* single deletion strain. An improved growth rate was observed in the *proV ptsP nagC* triple deletion mutant under this growth condition. A list of the gene disruption mutations that were found to provide increased tolerance to HMDA is shown in Table 21, and tested mutants that did not improve growth are listed in Table 22.

All Keio collection strains with knockouts in genes that were found to be mutated in Table 2 were screened for growth against the BW25113 control in M9 + 1% glucose + 32 g/L and also plus 38 g/L HMDA in the Growth Profiler screening format. Averaged growth curves for 3 biological replicate cultures are shown individually for each strain at 32 g/L and 38 g/L (Table

20). Moderate to large improvements in growth rate and lag time was observed in the *mpl*, *rph*, and *ybeX* deletion strains in 38 g/L HMDA, and for the *rph* and *ybeX* deletion strains in 32 g/L HMDA.

Table 16 - Preliminary screening of predicted loss-of-function mutations found in evolved strains in different concentrations of HMDA

	19 g/L HMDA				38 g/L HMDA			
	mean (1)		std. error (1)		mean (1)		std. error (1)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,550	2,1	0,035	0,5	0,119	33,1	0,011	2,1
MG1655 proV::kan	0,633	2,2	0,022	0,3	0,184	28,6	0,064	6,0
MG1655 ptsP::kan	0,622	2,2	0,023	0,1	0,138	22,8	0,011	0,9
MG1655 wbbK::kan	0,509	2,2	0,026	0,2	0,133	34,5	0,034	4,4
MG1655 ΔproV nagC::kan	0,543	1,8	0,038	0,3	0,000	-	0,000	-
MG1655 ΔproV ptsP::kan	0,729	2,6	0,035	0,3	0,299	33,0	0,016	3,0
	mean (2)		std. error (2)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,451	7,5	0,017	0,2	0,134	47,4	0,024	0,9
MG1655 proV::kan	0,545	6,8	0,051	0,1	0,194	36,5	0,041	2,6
MG1655 ptsP::kan	0,520	6,8	0,020	0,1	0,154	37,4	0,039	2,4
MG1655 wbbK::kan	0,478	7,3	0,016	0,1	0,101	42,5	0,011	0,7
MG1655 ΔproV nagC::kan	0,525	7,6	0,018	0,1	0,000	-	0,000	-
MG1655 ΔproV ptsP::kan	0,658	6,4	0,009	0,1	0,331	37,2	0,007	2,1

Table 17 - Second preliminary screening of predicted loss-of-function mutations found in evolved strains in different concentrations of HMDA

	19 g/L HMDA				38 g/L HMDA			
	mean (1)		std. error (1)		mean (1)		std. error (1)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,569	4,0	0,011	0,2	0,197	2,4	0,053	4,8
MG1655 proV::kan	0,586	4,1	0,028	0,3	0,307	3,3	0,026	1,9
MG1655 ptsP::kan	0,558	4,3	0,029	0,9	0,284	5,0	0,022	1,1
MG1655 wbbK::kan	0,575	3,8	0,081	0,6	0,209	7,3	0,026	8,3
MG1655 ΔproV ptsP::kan	0,667	3,5	0,030	0,2	0,308	3,7	0,022	0,7
MG1655 ΔproV wbbK::kan	0,650	3,5	0,040	0,2	0,317	5,4	0,018	0,8
MG1655 ΔproV nagC::kan	0,599	3,7	0,025	0,4	0,279	3,4	0,014	0,2
MG1655 ΔproV ΔptsP wbbK::kan	0,732	3,7	0,027	0,2	0,320	11,9	0,019	3,7

MG1655 ΔproV ΔptsP nagC::kan	0,630	3,8	0,029	0,1	0,247	5,8	0,016	1,8

	mean (2)		std. error (2)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,496	7,7	0,004	0,0	0,130	19,8	0,002	0,9
MG1655 proV::kan	0,553	8,8	0,025	0,1	0,238	17,8	0,013	0,1
MG1655 ptsP::kan	0,554	8,7	0,033	0,9	0,232	17,5	0,004	1,7
MG1655 wbbK::kan	0,479	8,5	0,022	1,9	0,141	18,8	0,005	4,4
MG1655 ΔproV ptsP::kan	0,619	6,6	0,024	0,0	0,343	13,6	0,004	0,1
MG1655 ΔproV wbbK::kan	0,612	7,0	0,008	0,1	0,256	15,4	0,007	1,1
MG1655 ΔproV nagC::kan	0,525	7,1	0,011	0,0	0,216	15,7	0,004	0,5
MG1655 ΔproV ΔptsP wbbK::kan	0,571	6,6	0,047	0,2	0,298	17,7	0,001	1,8
MG1655 ΔproV ΔptsP nagC::kan	0,537	7,1	0,008	0,1	0,238	20,3	0,002	0,9

Table 18 - First Biolector growth screen of loss-of-function mutants inferred from HMDA evolved isolates

	mean (1)		std. error (1)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,081	39,4	0,004	4,8	0,099	37,1	0,009	1,4
HMDA1-10	0,370	5,7	0,031	0,8	0,407	7,5	0,003	0,3
HMDA3-5	0,504	7,7	0,021	0,7	0,627	10,0	0,033	0,7
HMDA5-4	0,516	10,5	0,023	1,3	0,710	13,3	0,033	1,5
HMDA7-1	0,522	5,3	0,007	0,5	0,397	5,2	0,012	0,2
MG1655 proV::kan	0,174	13,9	0,001	4,6	0,205	19,0	0,005	0,9
MG1655 ptsP::kan	0,174	13,1	0,005	3,6	0,208	17,7	0,003	1,2
MG1655 wbbK::kan	0,112	29,7	0,004	2,4	0,128	26,2	0,005	1,3
MG1655 ΔproV nagC::kan	0,105	33,7	0,005	5,9	0,139	33,2	0,013	4,4
MG1655 ΔproV ptsP::kan	0,208	20,6	0,016	1,6	0,216	22,1	0,013	1,4
MG1655 ΔproV wbbK::kan	0,135	29,5	0,040	7,0	0,149	29,2	0,054	1,3
MG1655 ΔproV ΔptsP wbbK::kan	0,213	12,7	0,015	5,3	0,250	17,5	0,023	1,6
MG1655 ΔproV ΔptsP nagC::kan	0,248	25,6	0,027	4,1	0,297	25,5	0,000	1,3

Table 19 - Second Biolector growth screen of loss-of-function mutants inferred from HMDA evolved isolates

	mean (1)		std. error (1)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,098	33,3	0,029	5,3	0,078	22,9	0,028	10,0

HMDA1-10	0,392	6,6	0,045	1,1	0,407	7,7	0,034	0,7
HMDA3-5	0,527	10,4	0,016	0,4	0,670	11,5	0,015	0,3
HMDA5-4	0,522	13,1	0,014	0,3	0,754	15,4	0,097	0,2
HMDA7-1	0,441	6,0	0,038	0,1	0,419	6,1	0,020	0,7
MG1655 proV::kan	0,145	18,1	0,009	0,6	0,124	16,7	0,017	1,5
MG1655 ptsP::kan	0,159	18,4	0,004	1,8	0,153	18,4	0,011	1,5
MG1655 wbbK::kan	0,095	26,5	0,004	3,1	0,088	19,4	0,013	1,6
MG1655 ΔproV nagC::kan	0,000	-	0,000	-	0,154	58,0	0,008	4,0
MG1655 ΔproV ptsP::kan	0,137	23,5	0,047	4,5	0,155	20,1	0,011	0,1
MG1655 ΔproV wbbK::kan	0,117	28,2	0,018	8,1	0,114	23,4	0,012	2,0
MG1655 ΔproV ΔnagC ptsP::kan	0,173	25,8	0,062	6,0	0,227	23,6	0,025	1,1
MG1655 ΔproV ΔptsP wbbK::kan	0,148	28,4	0,034	11,5	0,165	20,6	0,022	0,1

Table 20 - Keio collection mutants exhibiting qualitatively improved growth in preliminary Growth Profiler screening (either with 32 g/L or 38 g/L HMDA) grown in 32 g/L or 38 g/L HMDA

	32 g/L HMDA				38 g/L HMDA			
	mean (1)		std. error (1)		mean (1)		std. error (1)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
BW25113	0,329	4,6	0,021	0,8	0,136	22,0	0,009	3,1
BW25113 ybeX::kan	0,422	3,8	0,068	0,3	0,268	6,0	0,038	2,2
BW25113 mpl::kan	0,287	5,1	0,011	2,0	0,129	10,8	0,009	6,8
BW25113 pstB::kan	0,356	3,7	0,057	1,2	0,107	32,8	0,023	0,0
BW25113 rph::kan	0,334	3,4	0,007	0,2	0,122	18,5	0,010	2,6
	mean (2)		std. error (2)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
BW25113	0,271	14,7	0,005	0,2	0,105	46,3	0,025	1,4
BW25113 mpl::kan	0,232	16,6	0,029	2,0	0,119	38,2	0,014	2,4
BW25113 pstB::kan	0,260	14,1	0,014	0,7	0,110	43,7	0,038	4,0
BW25113 rph::kan	0,329	12,7	0,006	0,2	0,131	38,0	0,023	0,0
BW25113 ybeX::kan	0,387	12,0	0,011	0,1	0,179	19,9	0,002	0,3

Table 21 - Gene deletion mutants exhibiting improved growth in high concentrations of HMDA

Strain genotype	Improved growth parameter
K-12 MG1655 *proV::kan*	Growth rate and lag time
K-12 MG1655 *ptsP::kan*	Growth rate and lag time
K-12 MG1655 *wbbK::kan*	Growth rate and lag time
K-12 MG1655 Δ*proV ptsP::kan*	Growth rate and lag time
K-12 MG1655 Δ*proV* Δ*ptsP wbbK::kan*	Growth rate and lag time

K-12 MG1655 Δ*proV* Δ*ptsP nagC::kan*	Growth rate and lag time
BW25113 *ybeX::kan*	Growth rate and lag time
BW25113 *mpl::kan*	Growth rate and lag time
BW25113 *rph::kan*	Growth rate and lag time

Table 22 - Tested gene deletion mutants that did not exhibit improved growth (or improved growth over single or double mutants when in higher combinations)

Strain genotype	Growth rate improvement	Lag time improvement
K-12 MG1655 Δ*proV nagC::kan*	negative to neutral	negative to neutral
K-12 MG1655 Δ*proV wbbK::kan*	negative vs. Δ*proV*	negative vs. Δ*proV*
All available Keio collection strains with individual knockouts in genes shown in Table 6, except for those listed in Table 21	None	none

A summary of the genes discussed thus far with a description of the known gene function is included in Table 23.

Table 23 - Descriptions of genes disrupted in mutants with improved growth in high concentrations of putrescine and HMDA

Gene name	Description	Notes
proV	Glycine betaine/proline ABC transporter periplasmic binding protein	Other subunits of the same protein are ProW and ProX
cspC	Multicopy suppressor of mukB; cold shock protein homolog constitutively expressed at 37C; antitermination protein; affects rpoS and uspA expression	In the same operon as *yobF*
ptsP	PTS PEP-protein phosphotransferase Enzyme I (Ntr)	
wbbK	Involved in lipopolysaccharide biosynthesis	
yobF	DUF2527 family heat-induced protein, function unknown	In the same operon as *cspC*
nagC	N-acetylglucosamine-inducible nag divergent operon transcriptional repressor	In the same operon as *nagA*
rph	Pseudogene reconstruction, RNase PH	likely due to increased transcription of downstream *pyrE**
yicC	UPF0701 family protein, function unknown	In divergent operon from *rph*, may also be related to *pyrE* expression*
yjcF	Pentapeptide repeats protein, function unknown	
iscR	isc operon transcriptional repressor; suf operon transcriptional activator; icsR regulon regulator; oxidative stress- and iron starvation-inducible; autorepressor; contains Fe-S cluster	
yedP	Predicted mannosyl-3-phosphoglycerate phosphatase; function unknown; HAD19	
ybeX	Heat shock protein, putative Co2+ and Mg2+ efflux protein; contains two CBS domains	Beneficial for HMDA
mpl	UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase; recycles cell wall peptidoglycan	

* K-12 strains have a frameshift mutation in *rph* that decreases transcription of *pyrE* leading to known growth defect in minimal media

g) Investigation of causative point mutations

It was desired to investigate which coding mutations were also causative in a selection of the best-performing strains. Two putrescine-evolved isolates, PUTR3-1 and PUTR8-10, and two HMDA-evolved isolates, HMDA1-10 and HMDA7-1, were selected for performing conjugation-mediated genome shuffling with the wild-type background strain K-12 MG1655. This technique generates a library of mutants that undergo random transfers and recombination of segments of the genome of the evolved strains, allowing the possibility of isolating strains with only some portion of the original set of mutations that are also tolerant. One drawback of this technique is that mutations that are close to each other in the genome are frequently transferred together, and it can be difficult to effectively isolate mutants that underwent multiple conjugation events to transfer the required mutations.

Selected isolates that were obtained as described in the methods were grown in the Biolector with 38 g/L putrescine or HMDA and were whole-genome sequenced. The mutations present in each isolate (*e.g.* PUTR3-1_1) are annotated in the plots. New mutations that were not present in the evolved isolate are indicated in red, while mutations that were present in the original evolved isolate are shown in black (with the full genotype of the evolved isolate also displayed). It was decided to not resequence any isolates following conjugation with HMDA1-10, as growth screening revealed no isolates with a tolerance phenotype approaching that of HMDA1-10.

For PUTR3-1 (Table 24), most resequenced conjugants exhibit growth approaching the evolved isolate PUTR3-1, and all conjugants harbor the coding mutation in *ygaC* (R43L). Four out of 6 also harbor the mutation in the intergenic region between *edd* and *zwf*, including isolate PUTR3-1_12, which exhibits the highest growth rates of all conjugated isolates. Based on these results, it was decided to introduce the *ygaC* and *edd/zwf* point mutations into the *ΔproV* background strain (see next section), due to deletion in *proV* already having been shown to improve growth in putrescine.

For PUTR8-10 (Table 25), it appeared to be more difficult for multiple conjugation events to occur that would transfer all necessary mutations required for the PUTR8-10 phenotype to the wild-type background. A number of conjugated isolates clustered together with their growth behavior (PUTR8-10_1, 4, 6, 9, and 12), and all of these isolates harbored more mutations from PUTR8-10 than the PUTR8-10_10 isolate. It is possible that these poorer growing isolates harbor a combination of mutations (for example, they all have the intergenic

mutation between *pyrE* and *rph*) that reduces growth without the presence of every mutation in PUTR8-10. Because the #10 isolate exhibited the best growth, it was decided to introduce the *mreB* (A298V), *rpsG* (L157*), and *spoT* (R471H) mutations into the *ΔproV* background strain (see next section), due to deletion in *proV* already having been shown to improve growth in putrescine and because the frameshift mutation in *proX*, another subunit of the ProVWX ABC transporter, is extremely likely to be functionally equivalent to disrupting *proV*. It was also decided to reconstruct the *argG* non-coding point mutation.

For HMDA7-1 (Table 26), the majority of conjugated isolates exhibited growth behavior approximately equivalent to HMDA7-1. All strains exhibited a common core set of 3 mutations in *nusA* (L152R), *sspA* (F83C), and *rpsG* (L157*). As a result, it was decided to introduce these three mutations into the *ΔproV* background strain (see next section), due to deletion in *proV* already having been shown to improve growth in HMDA.

Table 24 - Biolector growth screen and detected variants following resequencing in K-12 MG1655 isolates that had been conjugated with strain PUTR3-1 and selected for growth on 38 g/L putrescine

		mean (1)		std. error (1)		mean (2)		std. error (2)	
strain	mutations	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	-	0,205	16,2	0,105	0,6	0,000	-	0,000	-
PUTR3-1	*mcrA/icdC* G→T, *edd/zwf* C→T, *ygaC* R43L, *proV* 1 bp ins., *rph/yicC* C→A, *spoT* R209H, *nusG* G166V, *lexN* N163I	0,393	12,1	0,029	0,6	0,343	13,8	0,009	0,3
PUTR3-1_1	*edd/zwf* C→T, *fliK* 9 bp amp., *ygaC* R43L, *proV* 1 bp ins.	0,282	11,4	0,003	0,4	0,206	10,1	0,005	0,4
PUTR3-1_3	*iscR* L113F, *ygaC* R43L, *proV* 1 bp ins.	0,280	11,7	0,016	0,3	0,221	10,3	0,004	0,4
PUTR3-1_7	*edd/zwf* C→T, *ygaC* R43L, *proV* 1 bp ins.	0,243	11,9	0,001	0,2	0,201	10,8	0,008	0,1
PUTR3-1_10	*edd/zwf* C→T, *fliK* 9 bp amp., *iscR* L113F, *ygaC* R43L, *proV* 1 bp ins.	0,280	11,1	0,002	0,2	0,241	12,2	0,020	0,4
PUTR3-1_12	*edd/zwf* C→T, *ygaC* R43L	0,196	11,7	0,011	0,2	0,234	9,6	0,006	0,5
PUTR3-1_13	not resequenced	0,250	12,0	0,017	0,6	0,133	8,4	0,007	0,4

PUTR3-1_15	*fliK* 9 bp amp., *iscR* L113F, *ygaC* R43L, *proV* 1 bp ins.	0,248	11,9	0,010	0,8	0,191	9,2	0,027	0,9

Table 25 - Biolector growth screen and detected variants following resequencing in K-12 MG1655 isolates that had been conjugated with strain PUTR8-10 and selected for growth on 38 g/L putrescine

strain	mutations	mean (1)		std. error (1)		mean (2)		std. error (2)	
		μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	none	0,205	16,2	0,105	0,6	0,000	-	0,000	0,6
PUTR8-10	*sfmH* F11S, *nagC* 47 bp del., *proX* 1 bp ins., *argG* C→A, *mreB* A298V, *rpsG* L157*, *pyrE/rph* 1 bp del., *spoT* R471H	0,363	4,5	0,035	0,9	0,347	7,8	0,016	0,2
PUTR8-10_1	*proX* 1 bp ins., *argG* C→A, *mreB* A298V, *rpsG* L157*, *pyrE/rph* 1 bp del., *spoT* R471H	0,218	11,3	0,016	1,7	0,192	9,4	0,022	0,4
PUTR8-10_4	*argG* C→A, *mreB* A298V, *rpsG* L157*, *pyrE/rph* 1 bp del., *spoT* R471H	0,202	9,7	0,092	6,6	0,206	11,4	0,028	0,2
PUTR8-10_6	*argG* C→A, *mreB* A298V, *rpsG* L157*, *pyrE/rph* 1 bp del., *spoT* R471H	0,216	11,6	0,012	2,9	0,180	15,5	0,066	11,4
PUTR8-10_9	*proX* 1 bp ins., *argG* C→A, *mreB* A298V, *rpsG* L157*, *pyrE/rph* 1 bp del., *spoT* R471H	0,211	7,6	0,018	0,0	0,201	11,4	0,025	0,3
PUTR8-10_10	*proX* 1 bp ins., *mreB* A298V, *rpsG* L157*, *spoT* R471H	0,271	6,6	0,013	0,6	0,198	10,4	0,011	0,3

PUTR8-10_12	*proX* 1 bp ins., *argG* C→A, *mreB* A298V, *rpsG* L157*, *pyrE/rph* 1 bp del., *spoT* R471H	0,243	8,9	0,025	0,9	0,270	9,7	0,027	0,9

Table 26 - Biolector growth screen and detected variants following resequencing in K-12 MG1655 isolates that had been conjugated with strain HMDA7-1 and selected for growth on 38 g/L HMDA

strain	mutations	mean (1)		std. error (1)		mean (2)		std. error (2)	
		μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	none	0,000	-	0,000	-	0,000	-	0,000	-
HMDA7-1	*wbbK* 1 bp del., *argY/argV* 284 bp del., *nusA* L152R, *sspA* F83C, *rpsG* L157*	0,221	2,7	0,045	1,0	0,254	7,1	0,070	0,6
HMDA7-1_4	*wbbK* 1 bp del., *rfbB* D58Y, *argY/argV* 284 bp del., *nusA* L152R, *sspA* F83C, *rpsG* L157*	0,189	2,7	0,066	0,5	0,167	2,4	0,035	3,3
HMDA7-1_5	*nusA* L152R, *sspA* F83C, *rpsG* L157*	0,268	3,3	0,065	0,7	0,234	5,8	0,030	0,2
HMDA7-1_7	*argY/argV* 284 bp del., *nusA* L152R, *sspA* F83C, *rpsG* L157*	0,278	2,9	0,022	0,7	0,265	5,4	0,012	0,9
HMDA7-1_8	*nusA* L152R, *sspA* F83C, *rpsG* L157*	0,245	2,2	0,072	1,2	0,151	3,4	0,024	0,7
HMDA7-1_11	*argY/argV* 284 bp del., *nusA* L152R, *sspA* F83C, *rpsG* L157*	0,266	2,8	0,034	0,9	0,195	1,9	0,023	0,2
HMDA7-1_13	*argY/argV* 284 bp del., *nusA* L152R, *sspA* F83C, *rpsG* L157*, *yjiJ* IS150 ins.	0,286	3,2	0,048	1,1	0,226	5,7	0,016	2,0

h) Reconstruction and testing of mutants harboring point mutations

For PUTR3-1, the *ygaC* (R43L) and intergenic *edd/zwf* mutations were first introduced individually into K-12 MG1655 *ΔproV*, and next the combination of the two mutations was made also in the *ΔproV* background. These mutants were tested against K-12 MG1655 and PUTR3-1 in a growth screen in the Biolector testing format with 38 g/L putrescine (Table 27). It was evident that the edd/zwf mutation exhibited no discernible phenotype, while the *ygaC* and *ygaC* plus edd/zwf mutant strains exhibited an identical phenotype, thus we could assign the *ygaC* (R43L) mutation as causative and responsible for the majority of the growth improvement in this strain in high concentrations of putrescine. The growth rate is dramatically improved in this mutant over the K-12 MG1655 background but it is still a little lower than the original PUTR3-1 evolved strain. The *ygaC* mutation has also been constructed in K-12 MG1655.

For PUTR8-10, the *rpsG* (L157*), *argG* (non-coding), *mreB* (A298V), and *spoT* (R471H) mutations were first introduced individually into K-12 MG1655 *ΔproV* (and the *rpsG* mutation was also introduced individually into K-12 MG1655), and next the double combinations of the *mreB*, *spoT*, and *argG* mutations with the *rpsG* mutation were constructed. These mutants were tested against K-12 MG1655 and PUTR8-10 as described for PUTR3-1 (Table 27). Mutants harboring the *spoT* mutation by itself and in combination with the *rpsG* mutation could not grow in 38 g/L putrescine. Thus we can conclude that this mutation needs to be present together with other mutations in PUTR8-10 to either have a neutral or positive growth benefit. The *argG* mutation by itself afforded a moderately improved growth rate increase, while the *rpsG* and *mreB* afforded dramatically improved growth rates when present individually. For *rpsG* mutants, growth rate was equivalently improved in both the K-12 MG1655 and *ΔproV* background strains, indicating that disruption of *proV* afforded no additional growth advantage in the presence of these mutations (also suggested by the conjugated isolate results in the previous section). Both the *argG* and *mreB* double mutants with *rpsG* exhibited further improved growth characteristics, with the *rpsG* and *mreB* double combination being the best tested to date, with a growth rate and lag time approaching that of PUTR8-10. The triple combination of the *rpsG*, *mreB*, and *argG* mutations is being constructed and will be tested in the near future. It is believed that the continued growth of PUTR8-10 where the other mutants enter stationary phase may be a result of the *spoT* mutation, which encodes an enzyme that both synthesizes and hydrolyzes (p)ppGpp, an molecule that binds RNA polymerase and signals cells to undergo the stringent response. An impairing of the stringent response in PUTR8-10 would explain its continued growth when other cells stop growing and enter the stationary phase.

For HMDA7-1, the *nusA* (L152R), *sspA* (F83C) were attempted to be introduced individually into K-12 MG1655 *ΔproV*. The *rpsG* (L157*) mutant had already been constructed for investigating PUTR8-10 in both K-12 MG1655 and the *ΔproV* background. While the *sspA* (F83C) mutant could be readily constructed, it was not possible to isolate a *nusA* (L152R) mutant out of over 100 screened colonies. With a significant screening effort, it was possible to to isolate the *nusA* mutant in the strain already harboring the *sspA* mutant. Thus this mutation alone is likely greatly reducing fitness during MAGE and subsequent plating, which is performed using LB medium. Thus the *sspA* and *rpsG* single mutants (both in K-12 MG1655 and the *ΔproV* background strain) and *sspA nusA* double mutant in K-12 MG1655 ΔproV were tested for growth in the Biolector in 38 g/L HMDA (Table 28). Both the *sspA* mutant and the rpsG mutants exhibited greatly improved growth, with the ΔproV mutation affording a negligible additional growth benefit. The *nusA* and *sspA* double mutant strain exhibited dramatically improved growth over the sspA single mutant. Additional combinations with the *rpsG* mutation are currently being constructed and will be tested in the near future. A *nusA sspA rpsG* triple mutant which was validated to have received the *rpsG* mutation was found by Sanger sequencing to have an additional mutated base in the *nusA* locus, highlighting the instability of the *nusA* (L152R) mutation and probable negative fitness cost in LB medium.

A summary of point mutant strains that improve tolerance to putrescine and HMDA is provided in Table 29. Without being limited to theory, these are believed to not be complete losses-of-function. The strains that did not exhibit improved growth are also shown in Tables 27 and 28. Descriptions of the gene names and functions are provided in Table 29.

Table 27: Growth of tested point mutant strains in high concentrations of putrescine

	mean (1)		std. error (1)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,074	21,8	0,011	4,6	0,000	-	0,000	-
MG1655 proV::kan	0,094	18,2	0,026	0,8	0,091	14,7	0,015	9,0
PUTR3-1	0,317	11,3	0,021	0,5	0,345	13,4	0,013	0,8
MG1655 ΔproV ygaC*	0,221	12,7	0,017	0,3	0,251	12,9	0,005	0,8
MG1655 ΔproV edd/zwf*	0,106	16,3	0,014	4,9	0,100	14,4	0,022	0,1
MG1655 ΔproV ygaC* edd/zwf*	0,217	12,8	0,010	1,4	0,238	13,2	0,021	0,5
PUTR8-10	0,274	2,7	0,015	0,9	0,318	7,8	0,010	0,5
MG1655 rpsG*	0,253	2,7	0,032	4,2	0,230	9,8	0,013	0,7
MG1655 ΔproV rpsG*	0,216	3,2	0,014	1,3	0,182	7,4	0,021	1,4
MG1655 ΔproV mreB*	0,266	11,5	0,010	0,1	0,270	11,9	0,009	0,3
MG1655 ΔproV spoT*	0,000	-	0,000	-	0,000	-	0,000	-
MG1655 ΔproV argG*	0,132	13,4	0,018	3,7	0,124	13,5	0,033	2,6

MG1655 ΔproV rpsG* mreB*	0,235	6,4	0,006	1,9	0,267	8,0	0,004	0,7
MG1655 ΔproV rpsG* spoT*	0,119	2,0	0,007	3,2	0,094	8,4	0,022	5,2
MG1655 ΔproV rpsG* argG*	0,201	3,4	0,024	5,0	0,258	10,3	0,005	1,5

Table 28: Growth of tested point mutant strains in high concentrations of HMDA

	mean (1)		std. error (1)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,511	31,6	0,049	6,2	0,664	32,8	0,074	5,9
MG1655 proV::kan	0,453	26,1	0,098	4,0	0,605	27,8	0,155	4,2
HMDA7-1	0,541	4,7	0,074	0,3	0,387	4,5	0,017	0,8
MG1655 ΔproV rpsG*	0,276	8,2	0,016	1,1	0,271	12,0	0,001	1,6
MG1655 ΔproV sspA*	0,207	11,4	0,022	8,9	0,271	18,5	0,008	0,7
MG1655 ΔproV sspA* nusA*	0,316	6,5	0,009	4,5	0,289	9,7	0,007	1,2
MG1655 rpsG*	0,252	6,2	0,030	2,2	0,225	9,1	0,001	1,2

Table 29: Descriptions of genes disrupted in mutants with improved growth in high concentrations of putrescine and HMDA

Gene name	Description	Notes
ygaC	Function unknown; Fur regulon	
rpsG	30S ribosomal subunit protein S7, mutated stop codon	see note below*
argG	Argininosuccinate synthase	the last enzyme of arginine biosynthesis; arginine is an intracellular precursor for native putrescine production
mreB	Cell wall structural actin-like protein in MreBCD complex; mecillinam resistance protein	see note below**
sspA	DUF2527 family heat-induced protein, function unknown	In the same operon as *cspC*
nusA	N-acetylglucosamine-inducible nag divergent operon transcriptional repressor	In the same operon as *nagA*

* K-12 MG1655 has a mutation in *rpsG* relative to other *E. coli* strains that results in a lengthened ORF; evolution in various stress conditions appears to result in retruncation (via a premature stop codon). The neighboring W156* mutation has been observed in strains evolved to Na^+ (Wu *et al.*, *Appl. Environ. Microbiol.*, **80**:2880-2888, 2014) and in one of our p-coumarate evolved populations (COUM4). We have shown that for p-coumarate, the mutation is strongly selected for in the presence of p-coumarate but not in M9 glucose medium.

** Different coding mutations in MreB have previously been observed during evolutions on high NaCl concentrations (I336L, T171S, S185F, K96Q; Winkler *et al.*, *Appl. Environ. Microbiol.*, **80**:3729-3740, 2014). Other MreB mutations that we have isolated from putrescine evolutions are N34K, E212A. I24M, and H93N. None occurred in our HMDA evolved isolates.

i) Reconstruction of *ybeX* and *mpl* knockouts in existing most tolerant strains

The Keio collection screening hits in HMDA, *ybeX* and *mpl*, were constructed in K-12 MG1655 as single knockouts. Additionally, single *ybeX* or *mpl* knockouts or the combination of both the *ybeX* and *mpl* knockouts were constructed in K-12 MG1655 harboring the single *rpsG* (L157*) mutation, the *rpsG* (L157*) and *mreB* (A298V) mutations, the *ygaC* (R43L) mutation, the *nusA* (L152R) and *sspA* (F83C) mutations, and in the *proV cspC* and *proV ptsP* double knockout strains. All of these strains were tested in the Biolector growth testing format in M9 + 38 g/L putrescine and M9 + 38 g/L HMDA.

In 38 g/L putrescine (Table 30 and Table 31), it is apparent that the *ybeX* mutation does not improve growth by itself, while the *mpl* single knockout strain exhibits a moderately improved growth rate and greatly reduced lag time. The *ybeX* mutation similarly reduces or results in unchanged growth relative to the background controls when in combination with other beneficial mutations. The *mpl* mutation uniformly improves growth in combination with other beneficial mutations, with the exception of the *rpsG* (L157*) *mreB* (A298V) strain, where the growth rate was unchanged. It should also be noted that the *nusA* (L152R) and *sspA* (F83C) mutations, in addition to the evolved strain HMDA7-1, exhibit improved growth in 38 g/L putrescine in addition to HMDA, illustrating the cross-resistance of these strains and mutants across inhibitory concentrations of different polyamines in most cases.

In 38 g/L HMDA (Table 32 and Table 33), the *ybeX* mutation significantly improves growth by itself. The *mpl* single knockout also exhibits improved growth, although to a lesser extent than the *ybeX* knockout strain. Both the *ybeX* and *mpl* knockouts additively improve growth rates and lag times in background strains, and the combination of both the *ybeX* and *mpl* knockouts generally further improves growth over the single knockouts in either gene. It should also be noted that the evolved strains PUTR3-1 and PUTR8-10 and other reconstructed strains that were originally only tested in 38 g/L putrescine, also exhibit greatly improved growth in 38 g/L HMDA. In particular, strains K-12 MG1655 Δ*proV nusA* (L152R) *sspA* (F83C) Δ*ybeX mpl::kan* and K-12 MG1655 Δ*proV rpsG* (L157*) *mreB* (A298V) Δ*ybeX mpl::kan* exhibited similar growth rates and lag times to the evolved isolate HMDA7-1, illustrating a full reconstruction of the tolerance phenotype in evolved isolates via a combination of additive mutations from multiple isolates.

Table 30 – Growth rates and lag times of selected knockout/MAGE mutants in M9 + 38 g/L putrescine, as measured in the Biolector testing format.

	mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,075	23,8	0,018	1,7
HMDA7-1	0,204	7,4	0,016	0,9
MG1655 ΔproV rpsG*	0,234	9,4	0,010	0,7
MG1655 ΔproV nusA* sspA*	0,095	0,0	0,009	0,0
MG1655 ybeX::kan	0,058	12,2	0,017	10,7
MG1655 mpl::kan	0,116	7,5	0,004	0,8
MG1655 ΔproV rpsG* ybeX::kan	0,182	10,9	0,006	0,8
MG1655 ΔproV rpsG* mpl::kan	0,196	5,8	0,009	0,9
MG1655 ΔproV rpsG* ΔybeX mpl::kan	0,139	4,4	0,006	1,4
MG1655 ΔproV nusA* sspA* ybeX::kan	0,088	0,0	0,020	0,0
MG1655 ΔproV nusA* sspA* mpl::kan	0,138	1,1	0,029	1,6
MG1655 ΔproV nusA* sspA* ΔybeX mpl::kan	0,144	4,4	0,028	3,3
MG1655 ΔproV ptsP::kan	0,090	30,9	0,031	0,7
MG1655 ΔproV ΔptsP ybeX::kan	0,064	33,1	0,036	23,2
MG1655 ΔproV ΔptsP mpl::kan	0,133	18,2	0,019	1,3
MG1655 ΔproV ΔptsP ΔybeX mpl::kan	0,086	18,5	0,006	3,5

Table 31 – Growth rates and lag times of selected knockout/MAGE mutants in M9 + 38 g/L putrescine, as measured in the Biolector testing format.

	mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,081	25,1	0,053	10,3
PUTR3-1	0,339	13,6	0,013	0,9
MG1655 ΔproV ygaC*	0,229	12,8	0,021	0,2
MG1655 ΔproV ygaC* ybeX::kan	0,152	13,4	0,022	0,9
MG1655 ΔproV ygaC* mpl::kan	0,224	11,8	0,011	0,4
MG1655 ΔproV ygaC* ΔybeX mpl::kan	0,129	11,8	0,006	0,5
PUTR8-10	0,332	8,6	0,006	0,2
MG1655 ΔproV rpsG* mreB*	0,280	7,7	0,004	0,6
MG1655 ΔproV rpsG* mreB* ybeX::kan	0,269	10,0	0,010	0,6
MG1655 ΔproV rpsG* mreB* mpl::kan	0,273	9,5	0,005	0,3
MG1655 ΔproV rpsG* mreB* ΔybeX mpl::kan	0,242	10,0	0,009	0,9
MG1655 ΔproV cspC::kan	0,159	28,3	0,046	2,9
MG1655 ΔproV ΔcspC ybeX::kan	0,155	27,7	0,049	0,6
MG1655 ΔproV ΔcspC mpl::kan	0,192	17,7	0,017	1,7
MG1655 ΔproV ΔcspC ΔybeX mpl::kan	0,186	18,4	0,017	1,3

Table 32 – Growth rates and lag times of selected knockout/MAGE mutants in M9 + 38 g/L HMDA, as measured in the Biolector testing format.

	mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,092	42,7	0,034	1,9
HMDA7-1	0,419	6,2	0,007	0,6
MG1655 ΔproV rpsG*	0,249	12,3	0,007	0,7
MG1655 ΔproV nusA* sspA*	0,317	14,9	0,064	10,6
MG1655 ybeX::kan	0,171	6,2	0,005	0,9
MG1655 mpl::kan	0,131	23,6	0,010	0,5
MG1655 ΔproV rpsG* ybeX::kan	0,280	6,9	0,009	0,3
MG1655 ΔproV rpsG* mpl::kan	0,287	8,9	0,020	0,6
MG1655 ΔproV rpsG* ΔybeX mpl::kan	0,315	6,6	0,007	0,0
MG1655 ΔproV nusA* sspA* ybeX::kan	0,292	5,5	0,031	1,5
MG1655 ΔproV nusA* sspA* mpl::kan	0,341	11,9	0,027	0,7
MG1655 ΔproV nusA* sspA* ΔybeX mpl::kan	0,410	5,6	0,037	0,6
MG1655 ΔproV ptsP::kan	0,139	30,3	0,067	18,0
MG1655 ΔproV ΔptsP ybeX::kan	0,243	19,1	0,031	2,9
MG1655 ΔproV ΔptsP mpl::kan	0,306	23,8	0,003	2,8
MG1655 ΔproV ΔptsP ΔybeX mpl::kan	0,340	16,2	0,012	2,3

Table 33 – Growth rates and lag times of selected knockout/MAGE mutants in M9 + 38 g/L HMDA, as measured in the Biolector testing format.

	mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,108	29,3	0,003	2,7
PUTR3-1	0,461	21,0	0,006	0,7
MG1655 ΔproV ygaC*	0,256	18,6	0,005	1,1
MG1655 ΔproV ygaC* ybeX::kan	0,391	9,2	0,009	0,3
MG1655 ΔproV ygaC* mpl::kan	0,260	14,0	0,009	0,9
MG1655 ΔproV ygaC* ΔybeX mpl::kan	0,356	10,8	0,048	1,4
PUTR8-10	0,402	13,4	0,012	0,7
MG1655 ΔproV rpsG* mreB*	0,354	9,9	0,004	0,6
MG1655 ΔproV rpsG* mreB* ybeX::kan	0,414	8,5	0,005	0,5
MG1655 ΔproV rpsG* mreB* mpl::kan	0,324	10,5	0,000	0,1
MG1655 ΔproV rpsG* mreB* ΔybeX mpl::kan	0,405	8,7	0,020	0,5
MG1655 ΔproV cspC::kan	0,191	40,8	0,032	3,0

MG1655 ΔproV ΔcspC ybeX::kan	0,302	12,9	0,003	1,9
MG1655 ΔproV ΔcspC mpl::kan	0,258	26,4	0,002	3,4
MG1655 ΔproV ΔcspC ΔybeX mpl::kan	0,360	13,5	0,012	1,5

j) Flow cytometric analysis of cell morphology

Cell morphological changes are suspected in many putrescine evolved strains due to the common occurrence of mutations in MreB and other cell wall related genes (*e.g.* MrdB, MurA, McrA). MreB has a well-known role in forming cytosolic protein filaments that interact with the inner membrane, assisting in the maintenance of cylindrical cell shape. Mutations that disrupt *mreB* have most commonly been observed to result in spherical cells and often cell lysis. Other genes are more directly related to peptidoglycan synthesis and maintenance. Cell morphology in a population can be analyzed in a quantitative manner through the measurement of forward and side scattered light. Forward scatter is related to cell size, with higher forward scatter intensities correlated with larger cell dimensions. Side scatter is related to the cell refractive index, with increased side scatter intensities correlating with a higher order of internal complexity (for example, curvature of membrane structures). In ordinary wild-type cells, cell shape varies as a function of the phase of growth. Exponentially growing cells are typically longer and more cylindrical. Stationary phase cells are typically smaller and more spherical.

A preliminary analysis of PUTR3-1 (no known cell wall related mutations present), PUTR4-3 (coding mutations in MrdA), and PUTR8-10 (MreB A298V) in stationary phase cells grown in M9 + 1% glucose indicated a larger average cell size in PUTR8-10 and a smaller average cell size in PUTR4-3, with PUTR3-1 having an approximately equivalent cell size to wild-type cells. This is suggestive of the MrdA and MreB coding mutations having resulted in these phenotypes.

Additional screens of all sequenced putrescine evolved strains indicate similar results for some strains, with smaller cell size in *e.g.* the PUTR4, PUTR7-7 and PUTR7-9, and PUTR6-2 strains in stationary phase (which all harbor mutations in either mrdA and murA) and larger cell size in exponential phase than wild-type cells.

A flow cytometric screen was conducted for putrescine and HMDA-evolved isolates that were identified to have mutations in genes related to the cell wall or maintenance of cell shape. Forward scatter intensities are non-linearly correlated with cell size, and are shown for each isolate in Table 34. PUTR3-9, PUTR4-3, PUTR5-1, PUTR6-2, PUTR7-1, and PUTR7-7 all exhibited reduced exponential phase (in M9 medium) forward scatter intensities (FSC) as

compared with K-12 MG1655. These isolates were also analyzed by phase contrast microscopy during exponential phase in M9 medium and it was found that all strains with reduced FSC values exhibited a more elongated and narrower cell shape (Figure 1).

The MrdB-E254K mutation was introduced by MAGE into K-12 MG1655, and this strain was grown in 38 g/L putrescine in the Biolector testing format (Table 35). While it is difficult to capture the greatly improved growth profile observed in this strain in this table, it is apparent that the lag time was greatly improved. The strain also grew to a density nearly equivalent to PUTR4-3, whereas K-12 MG1655 remained at a very low cell density. Thus this mutation appeared to be one of the most causative mutations in the PUTR4-3 background. It was also apparent by phase contrast microscopy that the MrdB-E254K mutation alone reconstitutes the cell morphology found in PUTR4-3, demonstrating that the identified cell wall mutations are likely responsible for the morphological phenotypes observed in other isolates.

Table 34 – Forward scatter intensity of selected putrescine and HMDA evolved isolates containing cell wall or cell shape related mutations indicated, as measured by flow cytometry of exponential phase cultures grown in M9 medium.

		FSC intensity	
strain	cell wall mutation	average	std. error
MG1655	-	433,3	11,6
PUTR2-4	MreB-N34K	521,0	9,0
PUTR3-9	MreB-E212A	316,3	20,5
PUTR4-3	MrdB-E254K	322,7	4,2
PUTR5-1	MreB-I24M	339,7	9,2
PUTR6-2	MurA-G141A	330,0	19,7
PUTR7-1	MreB-H93N	342,7	7,8
PUTR7-7	MurA-Y393S	340,3	28,6
PUTR8-10	MreB-A298V	482,7	12,5
HMDA5-4	AmpC-I205T	466,7	3,2

Table 35 – Growth rates and lag times of K-12 MG1655, PUTR4-3, and K-12 MG1655 harboring the MrdA-E254K mutation in M9 + 38 g/L putrescine, as measured in the Biolector testing format.

	mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0,153	18,9	0,008	0,3
PUTR4-3	0,273	14,2	0,005	0,8
MG1655 mrdB*	0,151	9,2	0,013	0,9

k) Cross-compound tolerance testing

Every secondary screened evolved isolate from the putrescine and HMDA evolutions was grown in the presence of every other compound in the study as indicated in the Methods. The normalized tOD1(evolved strain)/tOD1(wild-type) are presented in Figures 2 and 3 for the putrescine and HDMA evolved isolates, respectively. Lower values are indicative of a larger improvement in growth of the evolved isolate (left column) in that chemical condition (top row), whereas higher values are indicative of a lower improvement or decrease in growth compared to the wild-type. Averaged ratios across conditions and strains shown at the right and bottom of the plot allow for overall by-chemical and by-strain trends to be observed. Strain names that are followed by an asterisk (*) were not re-sequenced, and strain names in italics were found to be hypermutator strains.

Generally, a wide range of patterns can be observed for growth of putrescine-evolved isolates in the different chemicals (see the Table in Figure 2). As expected, most strains (21 out of 24) exhibit improved growth in putrescine (although it should be noted that the growth improvements will be diminished at this reduced concentration of 32 g/L). Additionally, 21 evolved isolates exhibit improved growth in 40 g/L glutarate, 18 exhibit improved growth in 32 g/L HMDA, 19 exhibit improved growth in adipate, 18 exhibit improved growth in 1,2-propanediol, and 19 exhibit improved growth in NaCl. These conditions are the majority of the chemicals present at high concentrations (30-60 g/L), therefore we can conclude that many of the mutations in PUTR strains are more generally improving tolerance to osmotic stress. Many mutations in PUTR strains appear to be maladaptive for growth in the presence of butanol and 2,3-butanediol, in particular. PUTR6-7 was a notable exception, exhibiting improved growth in butanol and 2,3-butanediol and reduced growth in the majority of high osmolarity conditions. Overall, re-sequenced strains PUTR5-1, PUTR6-7, PUTR8-6, and PUTR8-10 exhibited the poorest cross-compound tolerance.

Generally similar patterns can be observed for HMDA-evolved isolates, with 18 (out of 24) strains exhibiting improved growth in glutarate, 21 with improved growth in putrescine, and all 24 with improved growth in adipate (Figure 3). A notable exception is that many HMDA strains exhibited poor growth in 1,2-propanediol and NaCl, and 21 strains exhibited greatly improved growth in p-coumarate (particularly populations 5 through 8). A large number of strains also exhibited improved growth in isobutyrate and octanoate, suggesting the possibility that many HMDA strains possess a general tolerance mechanism towards acids. Those HMDA strains that are tolerant to, for example p-coumarate, also have improved tolerance toward isobutyrate, hexanoate, or octanoate. Genome-wide association studies may correlate particular mutations with the observed growth phenotypes.

Additionally, each evolved isolate was tested for cross-tolerance toward other polyamines and amine-containing compounds of biotechnological interest. First, K-12 MG1655 was tested in the Growth Profiler screening format for growth in the presence of a range of concentrations of each compound: 1,3-diaminopropane, 1,5-diaminopentane (cadaverine), spermidine, citrulline, ethylenediamine, carnitine, and ornithine. Variable concentrations of these compounds elicited growth inhibition in *E. coli* K-12 MG1655 (Table 36). Agmatine was additionally tested, but was found to be non-toxic up to 50 g/L concentration. Based on these results, a screening concentration was selected for the evolved isolates for which wild-type cells could achieve at a growth rate of 0.2-0.3 h^{-1} (versus uninhibited growth at 0.7-0.9 h^{-1} in M9 glucose minimal medium). These concentrations were: 35 g/L 1,3-diaminopropane, 35 g/L cadaverine, 40 g/L spermidine, 80 g/L citrulline, 18 g/L ethylenediamine, and 10 g/L ornithine. Evolved isolates of putrescine and HMDA grown in additional chain length diamines (ethylenediamine, 1,3-diaminopropane, and cadaverine) and the native triamine metabolite spermidine are shown in Tables 37 through 40. The majority of evolved isolates exhibit greatly improved growth rates and often-reduced lag times in all of these compounds compared with K-12 MG1655. A notable exception were isolates from population HMDA7, which exhibited abolished growth in 18 g/L ethylenediamine and poor growth than the majority of evolved isolates (although improved over K-12 MG1655) in 1,3-diaminopropane. The compounds citrulline and ornithine are polyamine-containing amino acids. Again, the majority of putrescine and HMDA-evolved isolates exhibited improved growth rates in the presence of inhibitory levels of these compounds for wild-type K-12 MG1655 (Tables 37 through 40). Among the only exceptions were PUTR7-1 in citrulline and PUTR5-1 and PUTR6-2 in ornithine.

Table 36. Growth rates and lag times of K-12 MG1655 in varying concentrations of agmatine, ethylenediamine, carnitine, citrulline, and ornithine, as measured in the Growth Profiler testing format.

agmatine (g/L)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0.747	5.1	0.030	0.2
10	0.700	5.3	0.039	0.2
20	0.630	5.6	0.070	0.1
25	0.648	6.1	0.101	0.3
30	0.610	6.3	0.056	0.2
40	0.632	7.0	0.046	0.2
50	0.523	7.9	0.036	0.2
ethylenediamine (g/L)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0.810	6.1	0.038	0.3

5	0.689	7.0	0.052	0.3
10	0.614	8.5	0.015	0.2
20	0.152	32.9	0.017	1.9
25	0.000	-	0.000	-
carnitine (g/L)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0.885	6.0	0.052	0.3
5	0.786	6.6	0.024	0.2
10	0.545	7.5	0.019	0.3
20	0.000	-	0.000	-
citrulline (g/L)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0.855	6.3	0.028	0.4
25	0.759	6.7	0.030	0.2
30	0.702	7.0	0.023	0.3
40	0.677	7.5	0.084	0.6
45	0.508	8.6	0.149	0.1
50	0.538	9.2	0.082	0.2
60	0.437	10.4	0.099	0.1
75	0.355	12.4	0.083	0.2
ornithine (g/L)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0.783	7.1	0.042	0.3
5	0.439	8.2	0.028	0.4
10	0.260	17.1	0.005	0.8
20	0.214	44.2	0.014	2.8
25	0.150	53.2	0.015	4.7
30	0.076	82.6	0.009	1.5
40	0.000	-	0.000	-

Table 37. Growth rates and lag times of putrescine-evolved isolates in inhibitory concentrations (as shown) of ethylenediamine, 1,3-diaminopropane, cadaverine, and spermidine, as tested in the Growth Profiler testing format.

Table 37A:

	18 g/L ethylenediamine				35 g/L 1,3-diaminopropane			
	mean (2)		std. error (2)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.221	13.2	0.024	0.2	0.000	-	0.000	-
PUTR2-4	0.191	13.8	0.021	1.5	0.178	54.8	0.064	2.4
PUTR2-6	0.241	13.4	0.018	0.6	0.172	42.0	0.053	14.3
PUTR3-1	0.431	8.6	0.019	0.6	0.223	26.6	0.003	0.8
PUTR3-9	0.248	10.5	0.020	0.5	0.117	34.6	0.014	2.8

PUTR3-10	0.403	9.1	0.009	0.4	0.232	27.4	0.008	3.6
PUTR4-3	0.413	9.8	0.025	0.5	0.229	19.1	0.007	0.7
PUTR4-7	0.367	12.0	0.008	0.2	0.225	23.8	0.006	3.3
PUTR4-8	0.349	11.4	0.004	0.2	0.229	19.1	0.008	1.5
PUTR5-1	0.405	9.8	0.032	0.6	0.094	32.2	0.002	5.3
PUTR5-6	0.395	9.8	0.046	0.3	0.124	34.5	0.002	1.6
PUTR5-8	0.408	9.4	0.009	0.2	0.156	31.4	0.024	1.3
PUTR6-2	0.375	12.3	0.041	0.7	0.199	22.6	0.010	3.1
PUTR6-7	0.486	9.1	0.015	1.4	0.251	23.7	0.014	2.4
PUTR6-10	0.462	9.4	0.020	0.5	0.222	27.2	0.009	1.9
PUTR7-1	0.356	9.6	0.007	0.1	0.140	19.3	0.011	1.3
PUTR7-7	0.414	10.4	0.024	0.3	0.221	20.5	0.007	0.7
PUTR7-9	0.380	10.1	0.042	0.7	0.226	20.2	0.003	0.3
PUTR8-3	0.449	8.3	0.005	0.5	0.181	23.0	0.056	3.5
PUTR8-6	0.369	8.3	0.029	1.1	0.138	23.8	0.008	0.6
PUTR8-10	0.327	9.0	0.033	0.5	0.112	22.9	0.035	1.6

Table 37B:

	35 g/L cadaverine				40 g/L spermidine			
	mean (2)		std. error (2)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.189	17.1	0.010	0.7	0.229	16.5	0.038	0.8
PUTR2-4	0.345	13.7	0.043	0.7	0.214	16.0	0.023	0.6
PUTR2-6	0.346	12.1	0.017	2.5	0.209	14.8	0.004	2.5
PUTR3-1	0.477	10.0	0.024	0.1	0.369	11.3	0.011	0.0
PUTR3-9	0.343	11.9	0.004	0.2	0.232	15.2	0.023	0.6
PUTR3-10	0.469	10.2	0.011	0.4	0.385	11.1	0.007	0.5
PUTR4-3	0.434	9.4	0.003	0.1	0.357	10.6	0.011	0.3
PUTR4-7	0.448	10.2	0.024	0.5	0.363	11.7	0.010	0.5
PUTR4-8	0.432	9.9	0.010	0.4	0.344	11.2	0.020	0.3
PUTR5-1	0.329	12.7	0.006	0.3	0.215	14.0	0.042	0.6
PUTR5-6	0.367	10.3	0.037	0.4	0.350	11.2	0.012	0.0
PUTR5-8	0.355	10.3	0.018	0.1	0.338	11.2	0.012	0.2
PUTR6-2	0.391	10.6	0.021	0.3	0.336	12.6	0.015	0.3
PUTR6-7	0.449	10.0	0.013	0.6	0.350	11.2	0.009	0.6
PUTR6-10	0.408	9.5	0.047	0.3	0.365	11.5	0.021	0.7
PUTR7-1	0.352	10.5	0.077	0.3	0.302	11.3	0.015	0.2
PUTR7-7	0.342	10.4	0.015	0.2	0.336	11.6	0.004	0.1
PUTR7-9	0.376	9.9	0.024	0.1	0.327	11.8	0.003	0.4
PUTR8-3	0.467	8.7	0.043	0.0	0.319	10.9	0.026	0.7
PUTR8-6	0.437	8.8	0.074	0.6	0.308	10.5	0.007	0.7
PUTR8-10	0.488	8.6	0.025	0.2	0.300	10.3	0.012	0.1

Table 38. Growth rates and lag times of HMDA-evolved isolates in inhibitory concentrations (as shown) of ethylenediamine, 1,3-diaminopropane, cadaverine, and spermidine, as tested in the Growth Profiler testing format.

	18 g/L ethylenediamine				35 g/L 1,3-diaminopropane			
	mean (2)		std. error (2)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.162	15.8	0.019	0.4	0.000	0.0	0.000	0.0
HMDA1-10	0.339	10.1	0.021	0.1	0.210	17.3	0.019	0.7
HMDA2-1	0.224	17.7	0.057	3.0	0.076	26.2	0.008	0.9
HMDA2-8	0.165	13.7	0.020	1.3	0.073	25.3	0.004	0.8
HMDA3-4	0.308	12.3	0.041	0.3	0.106	41.3	0.046	18.5
HMDA3-5	0.319	12.4	0.012	0.5	0.000	-	0.000	-
HMDA3-6	0.276	13.3	0.054	0.6	0.083	40.0	0.081	2.4
HMDA4-2	0.286	18.5	0.013	0.3	0.118	20.4	0.004	0.9
HMDA4-6	0.298	18.8	0.008	0.7	0.167	18.8	0.014	0.7
HMDA4-9	0.212	18.1	0.019	4.6	0.139	18.6	0.023	0.6
HMDA5-4	0.406	12.5	0.020	0.6	0.000	-	0.000	-
HMDA5-5	0.439	12.9	0.027	0.5	0.000	-	0.000	-
HMDA5-10	0.439	16.9	0.020	0.7	0.100	29.2	0.030	2.0
HMDA6-3	0.312	11.9	0.006	0.9	0.195	34.8	0.051	1.5
HMDA6-7	0.350	11.1	0.008	0.5	0.174	33.6	0.020	2.3
HMDA7-1	0.000	-	0.000	-	0.054	29.5	0.020	0.7
HMDA7-7	0.000	-	0.000	-	0.055	31.0	0.005	2.5
HMDA7-10	0.000	-	0.000	-	0.062	30.8	0.013	0.9
HMDA8-5	0.203	24.9	0.104	7.8	0.134	23.3	0.023	0.4
HMDA8-9	0.104	16.7	0.090	14.6	0.130	23.1	0.023	1.3
HMDA8-10	0.158	29.4	0.062	5.2	0.106	19.4	0.037	2.0

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	35 g/L cadaverine				40 g/L spermidine			
	mean (2)		std. error (2)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.169	17.1	0.032	0.7	0.232	17.4	0.013	0.5
HMDA1-10	0.365	8.8	0.007	0.2	0.243	49.5	0.045	4.2
HMDA2-1	0.406	9.8	0.022	0.2	0.082	23.6	0.009	0.6
HMDA2-8	0.418	9.7	0.011	0.4	0.073	23.4	0.010	1.4
HMDA3-4	0.521	9.2	0.004	0.6	0.359	12.4	0.020	1.1
HMDA3-5	0.532	9.6	0.004	0.4	0.393	16.0	0.004	1.0
HMDA3-6	0.524	9.3	0.019	0.3	0.363	11.6	0.045	1.0
HMDA4-2	0.469	8.5	0.025	0.3	0.275	10.8	0.007	0.4

HMDA4-6	0.498	8.3	0.024	0.0	0.261	12.5	0.017	0.8
HMDA4-9	0.475	8.2	0.011	0.1	0.241	12.0	0.008	0.0
HMDA5-4	0.526	10.2	0.011	0.3	0.372	16.2	0.037	1.5
HMDA5-5	0.533	9.2	0.008	0.7	0.398	12.8	0.037	1.5
HMDA5-10	0.404	10.6	0.008	0.1	0.336	12.2	0.009	0.1
HMDA6-3	0.429	9.4	0.021	0.1	0.214	12.7	0.011	0.8
HMDA6-7	0.461	9.5	0.010	0.2	0.247	12.8	0.011	0.2
HMDA7-1	0.433	8.7	0.007	0.2	0.241	12.0	0.006	0.3
HMDA7-7	0.439	9.2	0.000	0.4	0.227	11.9	0.023	0.1
HMDA7-10	0.460	8.9	0.011	0.4	0.241	12.6	0.015	0.7
HMDA8-5	0.496	9.3	0.002	0.1	0.291	14.9	0.029	0.7
HMDA8-9	0.498	9.3	0.020	0.2	0.284	15.2	0.013	0.1
HMDA8-10	0.497	8.7	0.015	0.6	0.248	15.0	0.024	0.4

Table 39. Growth rates and lag times of putrescine-evolved isolates in inhibitory concentrations (as shown) of citrulline and ornithine, as tested in the Growth Profiler testing format.

	80 g/L citrulline				10 g/L ornithine			
	mean (2)		std. error (2)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.347	10.3	0.029	0.2	0.331	12.8	0.003	0.3
PUTR2-4	0.493	11.4	0.024	0.3	0.697	6.9	0.116	0.5
PUTR2-6	0.508	11.0	0.016	0.5	0.775	7.1	0.116	0.1
PUTR3-1	0.601	8.0	0.021	0.5	0.612	4.7	0.101	0.2
PUTR3-9	0.414	8.7	0.077	0.3	0.521	5.4	0.041	0.1
PUTR3-10	0.599	8.2	0.002	0.7	0.638	4.8	0.060	0.2
PUTR4-3	0.571	8.2	0.012	0.4	0.928	5.4	0.110	0.1
PUTR4-7	0.569	9.4	0.018	0.3	0.837	5.8	0.020	0.1
PUTR4-8	0.536	9.2	0.034	0.3	0.794	5.6	0.037	0.2
PUTR5-1	0.385	9.9	0.007	0.1	0.405	7.6	0.078	0.7
PUTR5-6	0.463	9.5	0.009	0.1	0.855	5.6	0.018	0.1
PUTR5-8	0.432	8.6	0.088	0.3	0.885	5.5	0.008	0.1
PUTR6-2	0.433	11.0	0.007	1.2	0.391	17.1	0.050	0.7
PUTR6-7	0.575	8.7	0.014	0.8	0.938	4.8	0.040	0.3
PUTR6-10	0.534	8.5	0.003	0.0	0.864	5.2	0.018	0.2
PUTR7-1	0.202	13.1	0.058	1.1	0.659	6.1	0.029	0.0
PUTR7-7	0.471	11.1	0.004	0.1	0.865	5.8	0.028	0.1
PUTR7-9	0.460	11.4	0.030	0.5	0.782	5.5	0.052	0.1
PUTR8-3	0.542	7.9	0.031	0.4	0.724	4.9	0.099	0.2
PUTR8-6	0.574	7.5	0.016	0.2	0.720	4.7	0.025	0.2
PUTR8-10	0.575	8.0	0.008	0.6	0.720	4.9	0.037	0.1

Table 40. Growth rates and lag times of HMDA-evolved isolates in inhibitory concentrations (as shown) of citrulline and ornithine, as tested in the Growth Profiler testing format.

	80 g/L citrulline				10 g/L ornithine			
	mean (2)		std. error (2)		mean (2)		std. error (2)	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.296	12.2	0.007	0.1	0.375	13.3	0.020	0.4
HMDA1-10	0.601	9.1	0.019	0.1	0.764	6.3	0.047	0.1
HMDA2-1	0.445	11.6	0.103	1.4	0.725	6.0	0.018	0.3
HMDA2-8	0.516	9.8	0.008	0.2	0.760	5.6	0.013	0.1
HMDA3-4	0.637	11.3	0.013	0.7	0.911	7.8	0.031	0.3
HMDA3-5	0.547	10.1	0.167	0.2	0.835	5.9	0.006	0.1
HMDA3-6	0.655	10.7	0.030	0.2	0.901	8.0	0.033	0.1
HMDA4-2	0.561	16.2	0.021	0.6	0.814	10.3	0.058	0.3
HMDA4-6	0.525	16.7	0.116	0.9	0.801	12.5	0.070	0.6
HMDA4-9	0.583	13.0	0.005	2.8	0.761	8.5	0.031	0.9
HMDA5-4	0.627	9.5	0.089	0.4	0.750	5.6	0.013	0.1
HMDA5-5	0.654	9.6	0.015	0.2	0.783	4.9	0.035	0.1
HMDA5-10	0.513	11.7	0.021	0.2	0.818	6.5	0.094	0.4
HMDA6-3	0.402	10.8	0.255	3.1	0.852	5.2	0.081	0.3
HMDA6-7	0.565	8.2	0.006	0.4	0.810	5.3	0.030	0.1
HMDA7-1	0.518	27.1	0.010	1.0	0.810	7.1	0.012	0.1
HMDA7-7	0.462	27.7	0.103	1.0	0.810	7.3	0.009	0.1
HMDA7-10	0.507	27.7	0.007	1.5	0.807	7.2	0.014	0.2
HMDA8-5	0.487	15.8	0.012	0.7	0.715	6.3	0.034	0.1
HMDA8-9	0.463	16.8	0.088	1.5	0.746	6.4	0.106	0.3
HMDA8-10	0.497	15.6	0.002	0.5	0.887	6.6	0.078	0.1

l) Biological production of polyamines

E. coli has been metabolically engineered to produce up to 24.2 g/L putrescine from glucose (Qian *et al.*, Biotechnol. Bioeng. 104:651-662, 2009). A schematic of the modifications employed in the overproducing *E. coli* K-12 W3110 strain is shown in Figure 1 of Qian *et al.* (2009), which figure is hereby specifically incorporated by reference. Briefly, the native *E. coli* pathway leading to L-ornithine was employed, with the ArgB, ArgC, ArgD, and ArgE overexpressed by replacing the native promoter of the argBCDE operon with an inducible Ptrc promoter. The promoter for the speF-potE promoter was also replaced with an inducible Ptrc promoter, with PotE being a putrescine export protein. The native promoter for *speC*, encoding an ornithine decarboxylase responsible for converting L-ornithine to putrescine, was

also replaced with an inducible Ptrc promoter to increase its expression, and this gene was additionally overexpressed off a plasmid (p15SpeC). The *argI*, *speE*, *speG*, and *puuPA* operons were deleted from the genome to prevent putrescine conversion to other products, conversion of L-ornithine to L-arginine, and putrescine re-import via the PuuP importer. The best producing strain in fed-batch fermentations (XQ52/p15SpeC) also contained a deletion of *rpoS*, which encodes the stationary phase sigma factor.

XQ52 and p15SpeC were generously donated by S. Y. Lee (Qian *et al.*, 2009) and were used to conduct two types of screens for putrescine production. In the first screen, evolved isolates were transformed with p15SpeC to allow a low level of putrescine overproduction in an otherwise unmodified background strain. They were compared for putrescine overproduction with K-12 MG1655 harboring p15SpeC, and XQ52 harboring p15SpeC as a positive control in a batch screen as described in the Methods (Table 41. After 24 hours, a number of evolved isolates exhibited higher putrescine titers than the K-12 MG1655 control, most notably PUTR3-1, PUTR5-8, PUTR6-7, PUTR7-7, and PUTR7-9. After 48 hours, strains with the highest production were PUTR5-6, PUTR5-8, PUTR7-1, PUTR7-7, and PUTR7-9. This includes all isolates that contained the E575A mutation in RpoD (encoding the housekeeping sigma factor, sigma 70), indicating its causation in improved endogenous production of putrescine. PUTR5-6 and PUTR5-8 contained an additional mutation in RpoC (V401G), while PUTR7-7 and PUTR7-9 contained additional mutations in MurA (Y393S) and RpoB (R637L). Without being limited to theory, the MurA mutation was believed to be responsible for the reduced FSC values observed in Table 34. PUTR7-1, by contrast, harbored mutations in RpsA (D310Y), NusA (M204R), MreB (H93N), and SpoT (R467H). Without being limited to theory, the MreB-H93N mutation was also believed to be responsible for the reduced FSC values observed in Table 34.

Table 41 – Batch production screen for putrescine overproduction in XQ52, K-12 MG1655, and evolved isolates harboring plasmid p15SpeC, with titers measured after 24 and 48 hours cultivation.

	putrescine titer (g/L)	
strain	24 h	48 h
XQ52/p15SpeC	0,35	1,01
MG1655/p15SpeC	0,15	0,22
PUTR2-4/p15SpeC	0,18	0,31
PUTR2-6/p15SpeC	0,20	0,27
PUTR3-1/p15SpeC	0,30	0,32
PUTR3-9/p15SpeC	0,23	0,28
PUTR3-10/p15SpeC	0,22	0,34
PUTR4-3/p15SpeC	0,12	0,15

PUTR4-7/p15SpeC	0,18	0,19
PUTR4-8/p15SpeC	0,21	0,27
PUTR5-1/p15SpeC	0,13	0,18
PUTR5-6/p15SpeC	0,16	0,43
PUTR5-8/p15SpeC	0,29	0,41
PUTR6-2/p15SpeC	0,15	0,13
PUTR6-7/p15SpeC	0,25	0,23
PUTR6-10/p15SpeC	0,16	0,32
PUTR7-1/p15SpeC	0,07	0,44
PUTR7-7/p15SpeC	0,39	0,48
PUTR7-9/p15SpeC	0,35	0,39
PUTR8-3/p15SpeC	0,16	0,19
PUTR8-6/p15SpeC	0,19	0,24
PUTR8-10/p15SpeC	0,14	0,22

The best producing strains from Table 36 were grown in semi-batch cultivation with a glucose/ammonium sulfate/magnesium sulfate feed solution as described in Methods. In this condition, higher cell densities and putrescine titers were achieved (Table 42). The PUTR7-9 background exhibited the highest level of production (4.46 g/L compared to 3.73 g/L in K-12 MG1655), as well as the highest specific production normalized to cell density. The PUTR3-10 background also exhibited a slightly higher titer than K-12 MG1655.

Table 42 – Semi-batch production screen (with glucose/ammonium sulfate/magnesium sulfate feeding) for putrescine overproduction in K-12 MG1655 and selected evolved isolates harboring plasmid p15SpeC. Titers and specific production were measured after 48 hours cultivation.

	putrescine production at 48 h	
strain	g/L	g/L/OD_{600}
K-12 MG1655/p15SpeC	3,73	0,131
PUTR3-1/p15SpeC	3,67	0,118
PUTR3-10/p15SpeC	3,86	0,122
PUTR5-6/p15SpeC	2,32	0,082
PUTR5-8/p15SpeC	3,53	0,110
PUTR7-1/p15SpeC	2,85	0,129
PUTR7-7/p15SpeC	3,52	0,110
PUTR7-9/p15SpeC	4,46	0,141

In a second type of screen, the highly modified background strain XQ52 was modified by MAGE to introduce the most beneficial point mutations found for improving tolerance.

Plasmid p15SpeC was reintroduced into each background strain to generate the final production strain. In batch screening (Table 43, the *ygaC* and *sspA* mutant backgrounds were found to have slightly higher titers than XQ52 after 24 hours. After 48 hours, the *mreB*, *argG*, *rpsG*, and *rpsG argG* mutants exhibited the highest putrescine titers, with all mutants exhibiting higher titers than XQ52. In semi-batch cultivation with glucose/ammonium sulfate/magnesium sulfate feeding (Table 44, higher cell densities and putrescine titers were achieved, although they were again much lower than those published by Qian *et al.* (2009) and below exogenously toxic concentrations of putrescine. After 24 hours, the *argG* mutant exhibited a moderately increased titer compared with the XQ52 background. However after 48 hours, XQ52 exhibited the highest production.

Table 43 – Batch production screen for putrescine overproduction in evolved isolates PUTR4-3, PUTR8-10, XQ52, K-12 MG1655, and XQ52 harboring MAGE-generated tolerance mutations, containing plasmid p15SpeC. Titers were measured after 24 and 48 hours cultivation.

	putrescine titer (g/L)			
	mean		standard error	
strain	24 h	48 h	24 h	48 h
PUTR8-10/p15SpeC	0,49	0,43	0,03	0,10
PUTR4-3/p15SpeC	0,43	0,27	0,01	0,02
XQ52/p15SpeC	0,93	1,51	0,20	0,10
XQ52 argG*/p15SpeC	0,93	1,85	0,11	0,10
XQ52 mreB*/p15SpeC	0,66	1,87	0,08	0,02
XQ52 ygaC*/p15SpeC	0,97	1,80	0,05	0,05
XQ52 rpsG*/p15SpeC	0,67	1,84	0,05	0,01
XQ52 sspA*/p15SpeC	1,07	1,63	0,01	0,01
XQ52 rpsG* argG*/p15SpeC	0,65	1,84	0,05	0,06
XQ52 rpsG* mreB*/p15SpeC	0,58	1,72	0,03	0,06

Table 44 - Semi-batch production screen (with glucose/ammonium sulfate/magnesium sulfate feeding) for putrescine overproduction in XQ52 and XQ52 with MAGE-generated tolerance mutations, harboring plasmid p15SpeC. Titers and specific production were measured after 48 hours cultivation.

	putrescine production at 24 h		putrescine production at 48 h	
strain	g/L	g/L/OD_{600}	g/L	g/L/OD_{600}
XQ52/p15SpeC	2,21	0,157	7,66	0,267
XQ52 argG*/p15SpeC	2,67	0,232	7,50	0,268
XQ52 mreB*/p15SpeC	1,83	0,208	4,74	0,251

XQ52 ygaC*/p15SpeC	1,91	0,203	6,31	0,237
XQ52 rpsG*/p15SpeC	1,81	0,226	6,07	0,244
XQ52 sspA*/p15SpeC	1,90	0,183	5,53	0,241
XQ52 rpsG* argG*/p15SpeC	1,42	0,170	5,57	0,230
XQ52 rpsG* mreB*/p15SpeC	1,05	0,158	5,72	0,242

Production of putrescine from the Gram-positive bacteria *Corynebacterium glutamicum*, with a maximum reported titer of 88 g/L, has been reported (Kind *et al.*, 2014; Jensen *et al.*, 2015; Nguyen *et al.*, 2015; Schneider *et al.*, 2012; Meiswinkel *et al.*, 2013). There has been intensive interest in employing this microorganism for the production of putrescine and other polyamines due to their derivation from L-glutamate and L-lysine, two amino acids that are almost exclusively produced at high titer in this organism.

Cadaverine is a 5-carbon diamine intermediate in chain length between putrescine and HMDA. It was not employed in our evolution experiments due to its high cost, however it is highly likely that many of the putrescine and HMDA evolved strains are cross-tolerant to cadaverine, and this will be tested in the future. Cadaverine is natively produced in *E. coli* and derives directly from L-lysine via CadA (lysine decarboxylase). It has been reported that up to 9.6 g/L was produced in fed-batched fermentations using engineered *E. coli* K-12 W3110 (Qian *et al.*, Biotechnol Bioeng. 108:93-103, 2011). The modifications to this strain were deletion of *speE*, *speG*, *puuA*, and *ygjG* which convert cadaverine to other products, and puuP, which re-imports cadaverine from the extracellular medium, as shown in Figure 1 of Qian *et al.* (2011), which is specifically incorporated by reference. Various genes in the pathway leading to L-lysine were overexpressed however only the replacement of the native dapA promoter with the Ptrc promoter was necessary to achieve the highest reported titer. CadA was additionally overexpressed off a plasmid (p15CadA). Attempts were made to reduce acetate production by deletion of *iclR*, however this modification did not improve cadaverine production.

Cadaverine has more successfully been produced in *Corynebacterium glutamicum*, with a maximum reported titer of 88 g/L by fed-batch fermentation (Kind *et al.*, Metab. Eng. 25:113-123, 2014). This was obtained in a pre-existing highly engineered lysine-overproducing strain that possessed various genome modifications resulting in deregulation and redirection of flux into lysine production. Additional modifications to this strain included the genome integration of a codon-optimized *E. coli ldcC* (an alternative lysine decarboxylase in *E. coli* to CadA), deletion of a *C. glutamicum* N-acetyltransferase that converts cadaverine to N-acetylcadaverine, deletion of *lysE* encoding the lysine exporter, and overexpression of a *C. glutamicum* permease responsible for exporting cadaverine.

A summary of known biological pathways for producing polyamines and other and monomers for the production of polymers is shown in Figure 2 of Chung *et al.* (2015), which is hereby incorporated by reference in its entirety. In addition, Chae *et al.* (2015) and Qian *et al.* (2009, 2011), also incorporated by reference in their entireties, have reported metabolic engineering of *E. coli* for the production of 1,3-diaminopropane, putrescine and cadaverine.

Finally, as to biological production of hexamethylenediamine, Figures 10, 11, 13, 20, 21, 22, 24, 25, and 26 of US patent application publication No. 2012/0282661 A1 (Genomatica Inc.), which are hereby incorporated by reference in their entireties, describe biological pathways leading to HMDA from different precursors. This publication describes a recombinant cell that can produce 6-aminocaproic acid, and a recombinant cell that comprises an enzyme with 2-oxoheptane-1,7-dioate aminotransferase activity, or 2-oxoheptane-1,7-dioate decarboxylase activity, and 6-aminocaproic acid is a precursor for HMDA via a few enzymatic steps. Additional examples are shown for production of HMDA via succinyl-CoA and acetyl-CoA, 4-aminobutyryl-CoA and acetyl-CoA, glutamate, glutaryl-CoA, pyruvate and 4-aminobutanal, and 2-amino-7-oxosubarate. Additional pathways describing routes to some of these precursors from natively occurring precursors are also described.

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EP 2 580 315 A2 (Cobalt Technologies Inc.)

CLAIMS

1. A bacterial cell comprising a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces expression of an endogenous gene selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, ybeX* and *mpl*, or a combination of any thereof.

2. The bacterial cell of claim 1, comprising a genetic modification which reduces expression of *ybeX, proV, cspC, ptsP, wbbK, mpl* or *rph.*

3. The bacterial cell of any one of the preceding claims, comprising genetic modifications which reduce the expression of

a) *proV* and at least one of *ptsP, cspC, mpl,* and *ybeX;*

b) *proV, ptsP,* and at least one of *mpl* and *ybeX;*

c) *proV, cspC,* and at least one of *mpl* and *ybeX;*

d) *ybeX* and at least one of *proV, ptsP, cspC,* and *mpl;*

e) *proV, ptsP, ybeX,* and *mpl;*

f) *proV, cspC, ybeX,* and *mpl.*

4. The bacterial cell of any one of the preceding claims, wherein the genetic modification comprises a knock-down or knock-out of the endogenous gene.

5. The bacterial cell of any one of the preceding claims, further comprising a mutation in at least one of YgaC, RpsG, MreB, NusA, SspA, MrdB, RpoD, RpoC, RpoB, MurA, RpsA, SpoT, *argG, rph* or the *pyrE/rph* intergenic region.

6. A bacterial cell comprising at least one mutation selected from YgaC-R43L, RpsG-L157*, MreB-A298V, MreB-N34K, MreB-E212A, MreB-I24M, MreB-H93N, NusA-L152R, NusA-M204R, SspA-F83C, SspA-V91F, MrdB-E254K, RpoD-E575A, RpoC-V401G, RpoC-V453I, RpoC-R1140C, RpoC-L120P, RpoB-R637L, RpoB-G181V, MurA-G141A, MurA-Y393S, RpsA-D310Y, RpsA-D310G, RpsA-D160V, RpsA-N313K, RpsA-N315K, RpsA-E427R, SpoT-R209H, SpoT-R467H, SpoT-R471H, SpoT-R488C, SpoT-G530C and *argG-C324A*.

7. The bacterial cell of any one of the preceding claims, wherein the genetic modification provides for an increased growth rate, a reduced lag time, or both, of the cell in at least one of putrescine, hexamethylenediamine (HMDA), 1,3-diaminopropane, spermidine, agmatine, cadaverine, ethylenediamine, citrulline, and ornithine as compared to the bacterial cell without the genetic modification.

8. The bacterial cell of any one of the preceding claims, comprising a recombinant biosynthetic pathway for producing at least one of putrescine, HMDA, 1,3-diaminopropane, spermidine, agmatine, cadaverine, ethylenediamine, citrulline, and ornithine.

9. The bacterial cell of any one of the preceding claims, comprising

a) a genetic modification which reduces expression of *proV,* and at least one mutation selected from RpsG-L157* and MreB-A298V, optionally wherein the aliphatic polyamine is putrescine;

b) a genetic modification which reduces expression of *proV,* and mutations RpsG-L157* and MreB-A298V, optionally wherein the aliphatic polyamine is putrescine;

c) a genetic modification which reduces expression of *proV,* and a mutation YgaC-R43L, optionally wherein the aliphatic polyamine is putrescine;

d) a genetic modification which reduces expression of *ybeX*, and at least one of *proV, ptsP, cspC,* and *mpl*, and at least one mutation selected from SspA-F83C, NusA-L152R, RpsG-L157*, YgaC-R43L, and MreB-A298V, optionally wherein the aliphatic polyamine is HMDA;

e) a genetic modification which reduces expression of *ybeX* and *mpl*, and mutations NusA-L152R and SspA-F83C, optionally wherein the aliphatic polyamine is HMDA;

f) a genetic mutation which reduces expression of *ybeX* and *mpl*, and mutations RpsG-L157* and MreB-A298V, optionally wherein the aliphatic polyamine is HMDA;

g) a genetic mutation which reduces expression of *ybeX* and *mpl*, and mutation YgaC-R43L, optionally wherein the aliphatic polyamine is HMDA.

10. The bacterial cell of any one of the preceding claims, which is of the *Escherichia*, *Bacillus*, *Ralstonia*, *Pseudomonas* or *Corynebacterium* genus, such as of the *Escherichia coli* species.

11. A process for preparing a recombinant *E. coli* cell for producing a polyamine, comprising genetically modifying an *E. coli* cell to

a) introduce a recombinant biosynthetic pathway for producing a polyamine; and

b) knock-down or knock-out at least one endogenous gene selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA*, *rph, ybeX* and *mpl,* and/or

c) introduce at least one mutation selected from YgaC-R43L, RpsG-L157*, MreB-A298V, MreB-N34K, MreB-E212A, MreB-I24M, MreB-H93N, NusA-L152R, NusA-M204R, SspA-F83C, SspA-V91F, MrdB-E254K, RpoD-E575A, RpoC-V401G, RpoC-V453I, RpoC-R1140C, RpoC-L120P, RpoB-R637L, RpoB-G181V, MurA-G141A, MurA-Y393S, RpsA-D160V, RpsA-D310Y, RpsA-D310G, RpsA-N313K, RpsA-N315K, RpsA-E427R, SpoT-R209H, SpoT-R467H, SpoT-R467L, SpoT-R471H, SpoT-R488C, SpoT-G530C and *argG*-C324A.

12. A process for improving the tolerance of an *E. coli* cell to at least one aliphatic polyamine selected from putrescine, HMDA, 1,3-diaminopropane, spermidine, agmatine, cadaverine, ethylenediamine, citrulline, and ornithine, comprising

a) genetically modifying the *E. coli* cell to knock-down or knock-out at least one endogenous gene selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA*, *rph, ybeX* and *mpl*;

b) preparing a population of *E. coli* cells comprising one or more mutations in at least one endogenous gene selected from *ygaC*, *rpsG*, *mreB*, *nusA*, *sspA*, *mrdA, rpoD, rpoC, rpoB, murA, rpsA, spoT,* and *argG*; and selecting any host cell which has an improved tolerance to at least one of putrescine, HMDA, 1,3-diaminopropane, spermidine, agmatine,cadaverine, ethylenediamine, citrulline, and ornithine; or

c) both a) and b).

13. A method for producing an aliphatic polyamine, comprising culturing the bacterial cell of any one of claims 1 to 10, the *E. coli* cell obtained by the method of claim 11, or the improved *E. coli* cell of claim 12, in the presence of a carbon source.

14. A bacterial cell comprising genetic modifications which reduce the expression of at least two endogenous genes selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA*, *rph, ybeX* and *mpl*.

15. A composition comprising putrescine, HMDA, cadaverine, spermidine, agmatine,1,3-diaminopropane, ethylenediamine, citrulline, or ornithine at a concentration of at least 10 g/L and a plurality of bacterial cells of the *Escherichia* genus which comprise

a) at least one genetic modification which reduces expression of an endogenous gene selected from the group consisting of *proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, ybeX* and *mpl*, or a combination of any thereof;

b) a mutation in at least one of *ygaC*, *rpsG*, *mreB*, *nusA*, *sspA, mrdA, rpoD, rpoC, rpoB, murA, rpsA, spoT* and *argG* which improves the tolerance of the bacterial cell to putrescine, HMDA, cadaverine,1,3-diaminopropane, spermidine, agmatine, ethylenediamine, citrulline, or ornithine; or

c) a combination of a) and b).

FIG. 1A

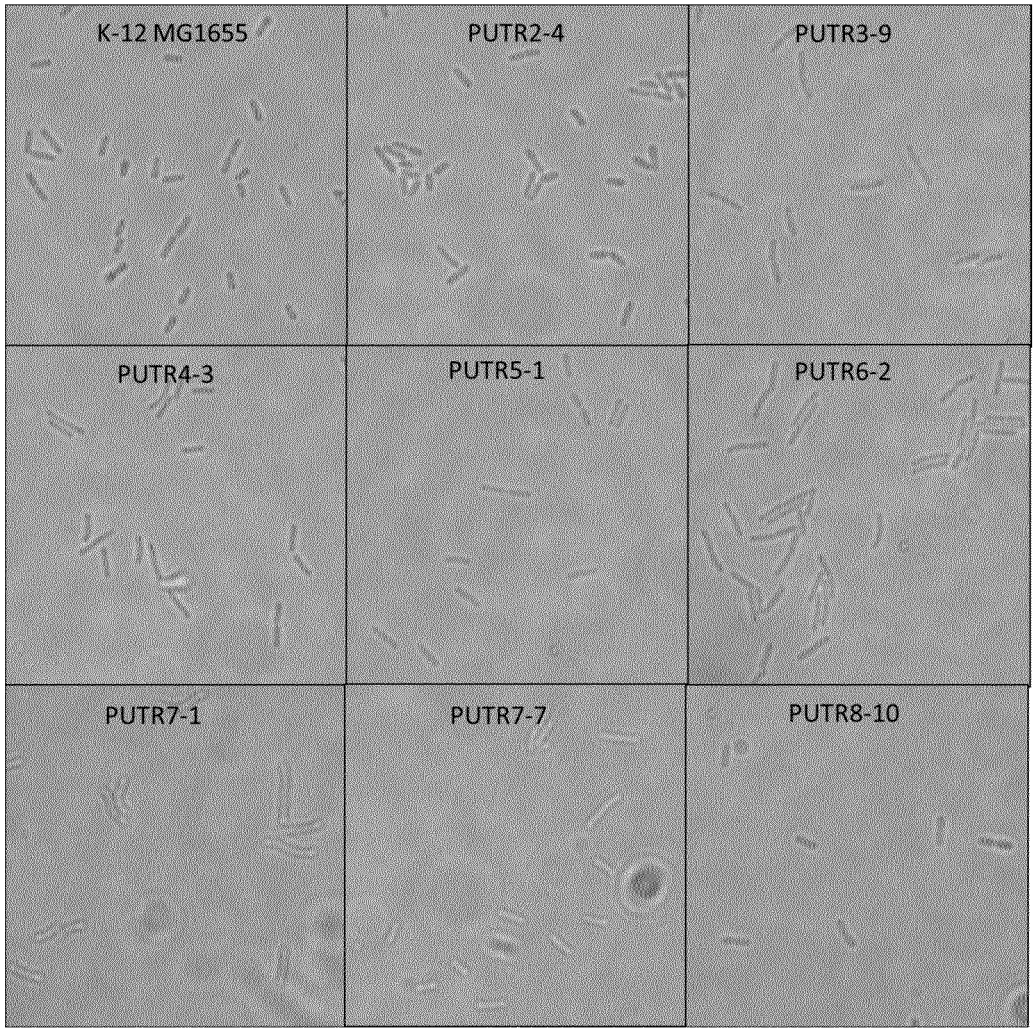
K-12 MG1655
PUTR2-4
PUTR3-9
PUTR4-3
PUTR5-1
PUTR6-2
PUTR7-1
PUTR7-7
PUTR8-10

FIG. 1B

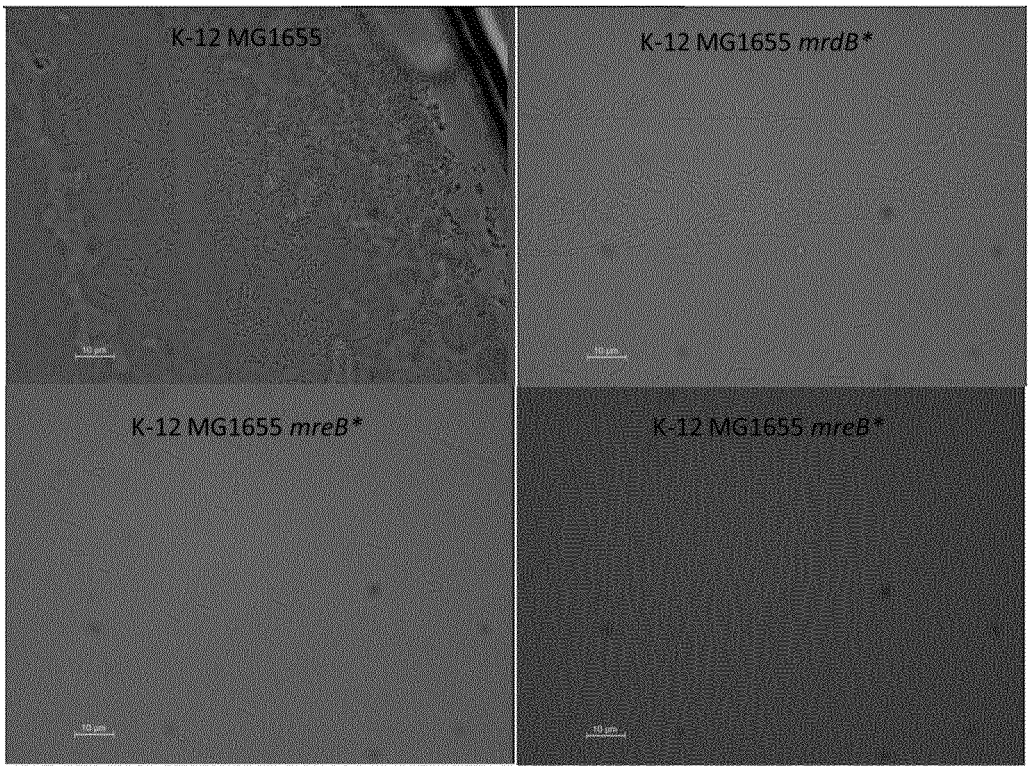

FIG. 2

	butanol	glutarate	coumarate	2,3-butanediol	putrescine	HMDA	adipate	isobutyrate	hexanoate	octanoate	1,2-propanediol	NaCl	average
PUTR1-3*	1,16	0,59	0,53	0,79	0,82	1,62	0,54	0,71	0,72	0,97	0,83	0,77	0,84
PUTR1-6*	1,16	0,59	0,51	0,69	0,70	1,45	0,59	0,69	0,62	0,95	0,74	0,83	0,79
PUTR1-9*	1,16	0,69	1,83	2,72	0,87	0,93	0,69	0,81	0,97	1,14	1,03	1,32	1,18
PUTR2-4	1,16	0,66	0,72	1,13	0,97	0,93	0,55	0,69	0,79	0,83	0,94	0,78	0,85
PUTR2-6	1,16	0,65	0,64	1,54	0,92	0,82	0,53	0,74	0,76	0,83	1,00	0,65	0,85
PUTR2-8*	1,16	0,69	0,69	1,42	0,87	0,89	0,55	0,74	0,79	0,79	0,94	0,78	0,86
PUTR3-1	1,16	0,68	0,62	0,73	0,67	0,59	0,51	0,88	0,76	0,63	0,60	0,71	0,71
PUTR3-9	1,16	0,71	0,82	0,87	0,78	1,11	0,72	0,74	0,86	1,80	0,83	0,80	0,93
PUTR3-10	1,16	0,65	0,60	0,66	0,65	0,60	0,52	1,14	0,79	0,72	0,74	0,63	0,74
PUTR4-3	1,16	0,62	1,04	0,82	0,72	0,59	0,52	0,74	0,69	1,25	0,83	0,62	0,80
PUTR4-7	1,16	0,72	1,37	0,80	0,73	0,62	0,61	0,76	0,69	1,80	0,80	0,57	0,89
PUTR4-8	1,16	0,71	1,68	0,73	0,73	0,62	0,65	0,71	0,66	1,71	0,83	0,71	0,91
PUTR5-1	1,16	1,03	1,54	1,41	0,92	1,10	1,18	1,86	1,76	0,84	1,51	1,46	1,31
PUTR5-6	1,16	0,82	0,86	1,13	0,77	0,63	0,72	0,88	0,86	1,00	0,97	0,72	0,88
PUTR5-8	1,16	0,81	0,56	1,04	0,75	0,56	0,76	0,93	0,86	1,05	0,94	0,54	0,83
PUTR6-2	1,16	0,75	0,79	1,01	0,88	0,88	0,69	1,24	1,03	1,44	0,97	0,83	0,97
PUTR6-7	0,81	1,35	1,67	0,76	1,60	1,59	1,42	2,90	2,10	0,96	0,94	1,95	1,51
PUTR6-10	0,89	0,78	0,64	0,77	0,70	0,64	0,59	1,29	0,93	0,79	0,83	0,52	0,78
PUTR7-1	1,16	1,16	0,56	0,82	0,67	0,64	1,16	1,05	1,14	0,77	0,91	0,91	0,91
PUTR7-7	1,16	0,84	1,03	2,03	0,73	0,51	1,08	0,86	1,07	1,30	1,03	0,88	1,04
PUTR7-9	1,16	0,78	1,01	2,72	0,72	0,53	1,02	0,81	0,97	1,10	0,97	0,85	1,05
PUTR8-3	0,92	0,57	0,95	0,85	0,62	0,66	0,52	1,14	1,38	0,64	0,91	0,80	0,83
PUTR8-6	1,16	0,90	1,42	2,72	1,12	0,79	0,76	1,45	1,45	0,92	1,51	1,15	1,28
PUTR8-10	1,16	0,84	1,45	2,72	1,95	1,64	0,78	1,48	1,38	0,92	1,60	2,89	1,57
average	1,12	0,77	0,98	1,29	0,87	0,87	0,74	1,05	1,00	1,05	0,97	0,94	0,97
# >wt	3	21	14	12	21	18	19	15	16	14	18	19	
% >wt	12,5	87,5	58,3	50,0	87,5	75,0	79,2	62,5	66,7	58,3	75,0	79,2	

FIG. 3

	butanol	glutarate	coumarate	2,3-butanediol	putrescine	HMDA	adipate	isobutyrate	hexanoate	octanoate	1,2-propanediol	NaCl	average
HMDA1-6*	0,83	0,55	0,72	1,10	0,72	1,06	0,43	0,86	0,70	0,74	5,36	2,61	1,31
HMDA1-8*	1,30	0,66	1,00	0,72	0,70	0,77	0,57	0,86	4,11	0,99	0,94	0,76	1,11
HMDA1-10	1,15	0,86	1,00	1,28	0,77	0,79	0,53	0,76	0,79	0,97	0,89	0,69	0,87
HMDA2-1	1,30	1,29	0,75	1,00	1,02	1,08	0,99	0,82	1,04	0,94	5,36	2,61	1,52
HMDA2-8	1,30	1,29	0,90	1,06	0,92	1,02	0,91	0,84	0,87	0,93	1,03	1,07	1,01
HMDA2-9*	1,28	1,32	0,74	1,06	0,97	1,02	0,88	0,84	0,91	0,94	5,36	2,61	1,49
HMDA3-4	1,30	0,65	0,55	1,73	0,77	0,83	0,51	0,69	0,72	0,96	0,97	0,77	0,87
HMDA3-5	1,15	0,57	0,37	0,88	0,79	0,81	0,44	0,71	0,79	0,91	5,36	2,61	1,28
HMDA3-6	1,30	0,62	1,00	1,60	0,75	0,83	0,52	0,69	0,87	0,94	1,22	0,78	0,93
HMDA4-2	1,30	1,28	0,76	1,14	0,75	0,81	0,54	0,86	1,09	1,10	1,11	0,81	0,96
HMDA4-6	1,06	1,14	0,81	1,11	0,85	1,00	0,74	0,86	1,13	1,07	5,36	1,26	1,37
HMDA4-9	1,30	1,18	0,62	0,89	0,69	0,79	0,48	0,90	1,47	1,01	1,00	0,72	0,92
HMDA5-4	1,05	0,63	0,31	0,78	0,85	0,90	0,48	0,76	0,91	0,98	5,36	2,61	1,30
HMDA5-5	1,11	0,65	0,49	0,84	0,90	0,96	0,57	0,82	1,02	0,93	0,92	0,89	0,84
HMDA5-10	1,30	0,83	0,34	0,94	1,00	1,15	0,70	1,20	1,06	1,84	2,56	2,61	1,29
HMDA6-3	0,83	0,65	0,45	0,83	0,75	0,98	0,59	0,69	1,00	0,83	1,19	0,78	0,80
HMDA6-7	0,94	0,65	0,33	1,52	0,75	0,83	0,54	0,73	0,77	0,95	1,25	0,84	0,84
HMDA6-10*	1,15	0,88	0,49	1,47	1,00	0,94	0,96	2,10	1,19	1,84	1,50	1,09	1,22
HMDA7-1	1,30	0,66	0,30	2,01	0,82	0,77	0,57	1,20	1,13	0,85	1,00	0,86	0,96
HMDA7-7	1,30	0,71	0,24	1,57	0,79	0,81	0,51	3,94	1,36	0,85	2,56	1,27	1,32
HMDA7-10	1,30	0,72	0,32	2,38	0,89	0,81	0,61	1,29	1,15	0,91	1,08	1,11	1,05
HMDA8-5	1,30	0,74	0,35	0,90	0,82	0,83	0,54	0,90	0,87	0,99	1,06	0,93	0,85
HMDA8-9	1,30	0,77	0,43	1,14	0,95	0,90	0,58	1,00	0,87	1,07	1,56	1,24	0,98
HMDA8-10	1,30	0,83	0,31	0,89	0,84	0,81	0,53	0,88	0,91	0,91	1,03	0,92	0,85
average	1,20	0,84	0,57	1,20	0,84	0,90	0,61	1,05	1,11	1,02	2,29	1,35	1,08
# >wt	3	18	21	9	21	18	24	18	12	18	4	12	
% >wt	12,5	75,0	87,5	37,5	87,5	75,0	100,0	75,0	50,0	75,0	16,7	50,0	

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/080059

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/00 C12N1/00 C12N1/20 C12N15/00 C12N15/01
C12N15/52 C12P13/00
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, EMBASE, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZHI-GANG QIAN ET AL: "Metabolic engineering of Escherichia coli for the production of putrescine, a four carbon diamine", BIOTECHNOLOGY AND BIOENGINEERING., vol. 104, no. 4, 1 November 2009 (2009-11-01), pages 651-662, XP055032553, US ISSN: 0006-3592, DOI: 10.1002/bit.22502 cited in the application abstract last complete sentence; page 659, left-hand column page 660, right-hand column, line 1 - line 6 page 661, left-hand column, line 13 - line 18 ----- -/--	1-5,7-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 February 2017	18/04/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Mundel, Christophe

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LI N ET AL: "Cadaverine production by heterologous expression of Klebsiella oxytoca lysine decarboxylase", BIOTECHNOLOGY AND BIOPROCESS ENGINEERING 2014 KOREAN SOCIETY FOR BIOTECHNOLOGY AND BIOENGINEERING KOR, vol. 19, no. 6, 2014, pages 965-972, XP002756871, ISSN: 1226-8372 abstract ----- DOKYUN NA ET AL: "Metabolic engineering of Escherichia coli using synthetic small regulatory RNAs", NATURE BIOTECHNOLOGY, vol. 31, no. 2, 20 January 2013 (2013-01-20), pages 170-174, XP055142093, ISSN: 1087-0156, DOI: 10.1038/nbt.2461 abstract page 172, right-hand column, line 31 - page 173, left-hand column, line 8 & Dokyun Na ET AL: "Metabolic engineering of Escherichia coli using synthetic small regulatory RNAs", Nature Biotechnology, vol. 31, no. 2, 20 January 2013 (2013-01-20), pages 170-174, XP055142149, ISSN: 1087-0156, DOI: 10.1038/nbt.2461 tables 2,3 ----- ZHI-GANG QIAN ET AL: "Metabolic engineering of Escherichia coli for the production of cadaverine: A five carbon diamine", BIOTECHNOLOGY AND BIOENGINEERING, vol. 108, no. 1, 30 January 2011 (2011-01-30), pages 93-103, XP055070575, ISSN: 0006-3592, DOI: 10.1002/bit.22918 cited in the application abstract ----- -/--	1-5,7-15 1-5,7-15 1-5,7-15

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JENS SCHNEIDER ET AL: "Improving putrescine production by Corynebacterium glutamicum by fine-tuning ornithine transcarbamoylase activity using a plasmid addiction system", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 95, no. 1, 28 February 2012 (2012-02-28), pages 169-178, XP035065837, ISSN: 1432-0614, DOI: 10.1007/S00253-012-3956-9 cited in the application abstract	1-5,7-15
A	LENNEN REBECCA ET AL: "Library sequencing strategies for comparative analysis of stress resistance mechanisms in Escherichia coli strains", NEW BIOTECHNOLOGY, vol. 31, no. Suppl. S, July 2014 (2014-07) , page S86, XP002756872, & 16TH EUROPEAN CONGRESS ON BIOTECHNOLOGY; EDINBURGH, UK; JULY 13 -16, 2014 the whole document	1-5,7-15
A	LENNEN REBECCA M ET AL: "Combinatorial Strategies for Improving Multiple-Stress Resistance in Industrially Relevant Escherichia coli Strains", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 80, no. 19, October 2014 (2014-10), pages 6223-6242, XP002756873, cited in the application abstract	1-5,7-15
A	SAXENA SHIVALIKA ET AL: "Compromised Factor-Dependent Transcription Termination in a nusA Mutant of Escherichia coli: Spectrum of Termination Efficiencies Generated by Perturbations of Rho, NusG, NusA, and H-NS Family Proteins", JOURNAL OF BACTERIOLOGY, vol. 193, no. 15, August 2011 (2011-08), pages 3842-3850, XP002766763, abstract	1-5,7-15

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/080059

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-4, 14(completely); 5, 7-13, 15(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-4, 14(completely); 5, 7-13, 15(partially)

   A bacterial cell comprising a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces the expression of an endogenous gene selected from the group consisting of proV, proW, proX, cspC, ptsP, wbbK, yobF, nagC, nagA, rph, ybeX and mpl, or a combination of any thereof.

1.1. claims: 1-4, 14(completely); 5, 7-13, 15(partially)

   A bacterial cell comprising a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces the expression of an endogenous gene selected from the group consisting of proV, proW and proX.

1.2. claims: 1-4, 14(completely); 5, 7-13, 15(partially)

   A bacterial cell comprising a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces the expression of the endogenous gene cspC.

1.3. claims: 1-4, 14(completely); 5, 7-13, 15(partially)

   A bacterial cell comprising a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces the expression of the endogenous gene ptsP.

1.4. claims: 1-4, 14(completely); 5, 7-13, 15(partially)

   A bacterial cell comprising a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces the expression of the endogenous gene wbbK.

1.5. claims: 1-4, 14(completely); 5, 7-13, 15(partially)

   A bacterial cell comprising a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces the expression of the endogenous gene yobF.

1.6. claims: 1-4, 14(completely); 5, 7-13, 15(partially)

   A bacterial cell comprising a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces the expression of the endogenous gene nagC.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1.7. claims: 1-4, 14(completely); 5, 7-13, 15(partially)

A bacterial cell comprising a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces the expression of the endogenous gene nagA.

1.8. claims: 1-4, 14(completely); 5, 7-13, 15(partially)

A bacterial cell comprising a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces the expression of the endogenous gene rph.

1.9. claims: 1-4, 14(completely); 5, 7-13, 15(partially)

A bacterial cell comprising a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces the expression of the endogenous gene ybeX.

1.10. claims: 1-4, 14(completely); 5, 7-13, 15(partially)

A bacterial cell comprising a recombinant biosynthetic pathway for producing an aliphatic polyamine and at least one genetic modification which reduces the expression of the endogenous gene mpl.

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2. claims: 6(completely); 5, 7-13, 15(partially)

A bacterial cell comprising at least one mutation selected from YgaC-R43L, RpsG-L157*, MreB-N34K, MreB-E212A, MreB-I24M, MreBH93N, NusA-L152R, NusA-M204R, SspA-F83C, SspA-V91F, MrdB-E254K, RpoD-E575A, RpoC-V401G, RpoC-V453I, RpoC-R1140C, RpoC-L120P, RpoB-R637L, RpoB-G181V, MurA-G141A, MurA-Y393S, RpsA-D310Y, RpsA-D310G, RpsA-D160V, RpsA-N313K, RpsA-N315K, RpsA-E427R, SpoT-R209H, SpoT-R467H, SpoT-R471H, SpoT-R488C, SpoT-G530C and argG-C324A.
